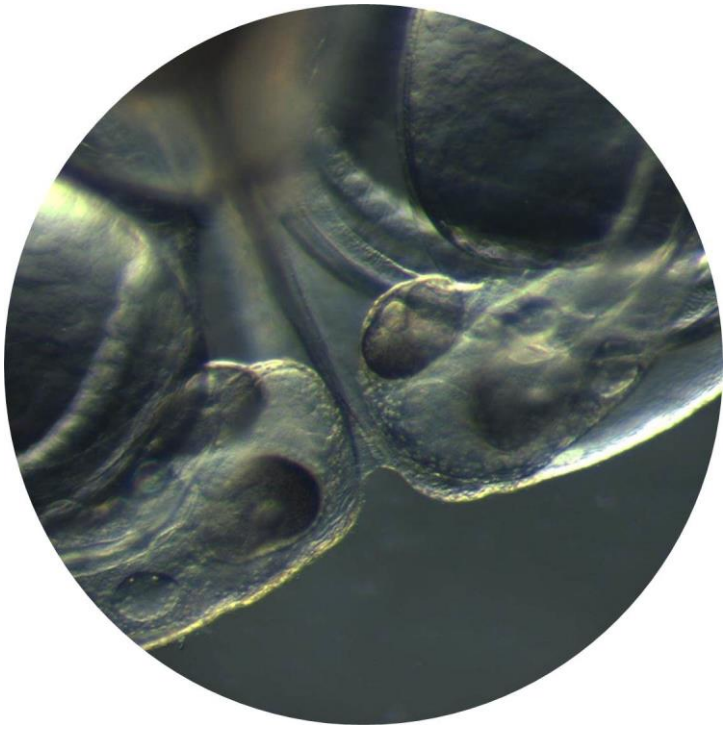
GEOMAR
Helmholtz Centre for Ocean Research Kiel

Local adaptation in Atlantic herring (*Clupea harengus)* - Interaction of salinity, pathogen and parents origin

- MASTER THESIS -



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30th November 2015

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1 Abstract

Habitat stratification induced by an abiotic factor (e.g. salinity) can initiate population divergence. Selection that promotes a fitness advantage in the local habitat can result in local adaptation of a population to the prevailing environmental conditions. Climate change will affect the Baltic salinity gradient and therefore also influence such evolutionary processes. The Atlantic herring (an economically important fish species) is known to be distributed across a wide salinity range and to show migratory behaviour between feeding and spawning grounds. Populations mix at feeding grounds, but during springtime populations migrate to different spawning grounds stratified by a salinity gradient. Hence, herring populations are supposed to be genetically structured according to the salinity level of their spawning grounds. The aim of this study was to experimentally evaluate local adaptation to the natural salinity gradient in two herring populations from the Baltic Sea (Kiel Canal and Little Belt, Denmark) and identify their potential of phenotypic plasticity. In addition, a second stressor (bacteria *Vibrio spp*.) was applied to evaluate the interaction of a biotic and abiotic (salinity) factor on local adaptation. The two populations had experienced different salinities at their spawning grounds. The gametes of wild adults were collected and crossed in all possible combinations (within and between population crosses). Fertilized eggs were exposed to native and novel salinity in a common garden approach. Four days post hatch the larvae were additionally exposed to a *Vibrio spp.* stress. As response variables life-history traits and gene expression were measured. I detected strong phenotypic plasticity in herring, with signs for ongoing local adaptation to the spawning ground in the Kiel Canal population. In the context of climate change, I suggest that not salinity decline will be the main stressor for Western Baltic herring, but rather another stressor that is affected by salinity. The increase of pathogen virulence (here *Vibrio spp*.) by decreasing salinity will probably be one of the main stressors. The suggestion of local adaptation to salinity on spawning sites in herring was until now solely based on neutral genetic markers. The results of this study give a new perspective based on phenotypic traits on the potential of local adaptation processes in Western Baltic herring.

2 Introduction

Natural environments are characterised by spatial and temporal variation in biotic and abiotic factors. If environments are stable, natural selection acts on traits providing a fitness advantage of a population in the respective habitat. If gene flow is limited among populations of a species and dispersal is low, the potential for local adaptation is enhanced (Kawecki and Ebert 2004). Local adaptation is defined by a genotype having a higher fitness in its native habitat (sympatric) compared to a foreign genotype from another population of the same species (allopatric) (Kawecki and Ebert 2004). Local adaptation can be an important step towards speciation (Schluter 2001). The marine environment is characterised by large spatial connectivity, with a potential of gene flow among all habitats (Palumbi 1994). This would speak for a rather low potential of population differentiation and local adaptation (Lenormand 2002). However, many studies showed examples of adaptive differentiation in the ocean across different taxa, e.g. invertebrates (reviewed by Sanford and Kelly 2011) and fishes (Conover 1998, Conover et al. 2006). Adaptive differentiation is driven by various biotic (e.g. predation, natural toxins and prey availability) and abiotic (e.g. salinity, temperature, pH, pollution and hypoxia) factors (reviewed by Sanford and Kelly 2011). Another important biotic factor driving local adaptation is the interaction of host and parasite (Kawecki and Ebert 2004), due to a constant evolutionary arms race between parasite virulence and host resistance (Hamilton 1980). As both, abiotic and biotic factors structure a habitat, local adaptation can be difficult to predict due to their interacting and potentially opposing effects. An example of such an interaction is the effect of salinity on pathogens. While the common marine *Vibrio* spp. bacteria are mostly opportunistic, they enhance their growth (Larsen 1984) and virulence (Wang 2005) under decreased salinity. Therefore, populations may not only be stressed due to challenges of coping and adapting to low saline waters, but they may also suffer from more frequent virulent infections.

Species currently adapted to local environmental conditions will face significant challenges in the future due to climate change, as populations have to adapt to novel conditions (Davis et al. 2005). Species can respond to changes through phenotypic plasticity (e.g. physiological, gene expression, behavioural), these plastic responses are solely a phenotype adjustment to environmental changes without genetic changes (Pigliucci 2001). Phenotypic plasticity can be an important way for populations to persist in a rapid changing environment, especially in displaying a source of novel opportunities and finally if such traits become genetically assimilated (Crispo 2008, Lande 2009). Parental effects can be seen as a component of phenotypic plasticity (trans-generational plasticity), as it is defined by the non-genetic transfer of information affecting the offspring's phenotype (Reusch et al. 2014). The general paradigm is that parental effects are mainly a female's attribute (e.g. egg quality in fish, Rideout 2005; transfer of antibodies, Gasparini et al. 2002), because the male gametes are too small to transfer other substances than DNA (Wassarman et al. 2001). In contrast to this assumption, Roth et al. (2010) found paternal trans-generational immune priming in invertebrates without brood care. A promising way for males to transfer such information is via genetic imprinting or the transfer of epigenetic factors, which can modify gene expression (Ashe

and Whitelaw, 2007). Parental effects on early life-stages of Atlantic cod (Kroll et al. 2013) and herring (Bang et al. 2006) were seen to affect larval length and mortality (paternal effect) and larval weight and yolk-sack volume (maternal effect). In the context of climate change, evaluating local adaptation and the potential of phenotypic plasticity of today's population will help us to predict how populations will cope with environmental changes. The importance of that knowledge has a mainly anthropogenic reason, as we depend to a large degree on marine resources for our food production (e.g. fisheries). Understanding local adaptation patterns is crucial for identifying protection areas that consider genetic diversity (Bell and Okamura et al. 2005). Locally adapted populations contain genotypes that may be able to cope with stressful conditions, therefore such population can act as source to replenish disturbed areas (McClanahan et al. 2007).

The Baltic Sea is an example for a highly stratified habitat due to the impact of an abiotic factor: salinity. The Baltic Sea is characterized by a salinity gradient having almost freshwater in the north and the east and an increasing salinity towards the south and the west. Beeing an almost land-locked basin with a tight connection to the more saline North Sea, the large river run-off of freshwater from the surrounding land masses is responsible for this salinity gradient (Segerstråle 1969). Climate change will have global effects on precipitation patterns (Trenberth 2011). Especially for the Baltic Sea a reduction in salinity is predicted for the next centuries due to increasing precipitation (Meier et al. 2006). This will affect the "horohalinicum" that will shift southward, leading to an expansion of the area with salinity less than 7 PSU and affecting species distribution and biodiversity (Vourinen et al. 2015; Fig. 1). The horohalinicum is the salinity range of five to seven, where the lowest number of species is found, as it also represents the tolerance border of fresh- and saltwater species (Kinne 1983).

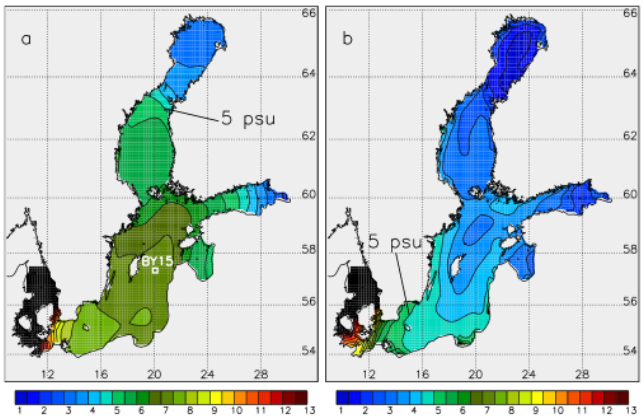


Fig. 1 From Meier et al. (2006) Left panel shows the sea surface salinity in the Baltic Sea today. Right panel shows a prediction for the year 2100. Indicated is the southward shift of the horohalinicum through the isoline five.

Populations of various taxa are distributed along the Baltic salinity gradient (Laine et al. 2003, Bonsdorff et al. 1999, Westerbom et al. 2002). As salinity is one of the major factors affecting species distribution in the Baltic Sea, projected changes in salinity will have strong impacts on the species composition. Especially in marine fish low salinity can be a problem for successful reproduction and development. Nissling et al. (2006) revealed decreased reproductive success in turbot from the Baltic Sea in salinities below 7 PSU. However, Baltic populations are known to have

adapted to the lower salinity compared to their fully marine counterpart. For example, cod eggs show an adaptation in their buoyancy level, which prevents them from sinking into oxygen-depleted depths (Petereit et al. 2014). Some of these adaptations resulted even in genetic differentiation, as for example in herring (Lamichhaney et al. 2012), turbot (Nielsen et al. 2004) and cod (Poćwierz-Kotus et al. 2015). How species distribution will shift, depends on the variation among taxa in the ability of adapting to the changing conditions. As already mentioned salinity has an impact on bacterial virulence (especially *Vibrio spp*.). With a drastic decline of salinity in the Baltic Sea due to climate change, virulence of *Vibrio spp.* may increase in the future. The question arises how these two factors may interact in respect to local adaptation. Selection to low salinity may be constrained by the high bacterial virulence and may hamper local adaptation.

Atlantic herring (*Clupea harengus*) is a key species in the Baltic Sea for ecological and economical (fisheries) reasons. In the Baltic Sea herring is distributed along a north-south salinity gradient, represented by the model from Jorgensen et al. (2005), which indicates that salinity and temperature rather than geographical distances correlate to genetic differentiation. In the transition zone between North Sea and Baltic Sea a steep salinity gradient is found, ranging from 30 PSU in the North Sea to 6 PSU in the Baltic proper. Studies using neutral genetic markers indicate that this area matches with a steep increase in genetic differentiation of various species, including Atlantic herring (Bekkevold et al. 2005). Herring in this region migrate from different spawning grounds to a common feeding ground and form seasonal aggregations of mixed populations (Ruzzante et al. 2006; Fig. 2). Though, Bekkevold et al. (2005) showed that these herring populations are genetically structured and, moreover, that this structure is related to the salinity variation in the spawning grounds. This indicates that population structure can be maintained in this migratory fish and that it is locally adapted to environmental conditions in spawning sites. The persistence of this intraspecific differentiation despite mixing in feeding grounds supports the assumption of natal homing in this species (Ruzzante et al. 2006). Additionally, Gaggiotti et al. (2009) suggest that gene flow from populations of high or low salinity grounds into populations of intermediate salinity is rare and that selection imposed by salinity is particularly high in early life stages of herring.

The intent of this study is to identify local adaptation to salinity at spawning grounds and the potential of phenotypic plasticity in two populations of Atlantic herring (*Clupea harengus*) spawning along the Baltic salinity gradient. By crossing two populations (within and between population crosses) and expose larvae to native and novel salinity in a common garden approach, I aim to address several hypotheses. As response variables served life-history traits and gene expression analyses. First, by comparing within population crosses among the different salinities I wanted to answer if populations from less saline habitats are better performing in their native salinity at spawning ground. Moreover, I wanted to elucidate if populations from less saline habitats are better adapted to a pathogen stress compared to populations from higher saline waters. With the exposure to a novel salinity, which none of the larvae in both population experienced in nature, I aimed to investigate the potential of phenotypic plasticity across populations. Secondly, by comparing within and between population crosses I wanted to disentangle the role (and effects) of mother and father in a system with no brood care and external fertilization in respect to the ability

to cope with the two stressors. In addition, if populations are locally adapted, the comparison with between population crosses should demonstrate a disruption of the local adaptation pattern.

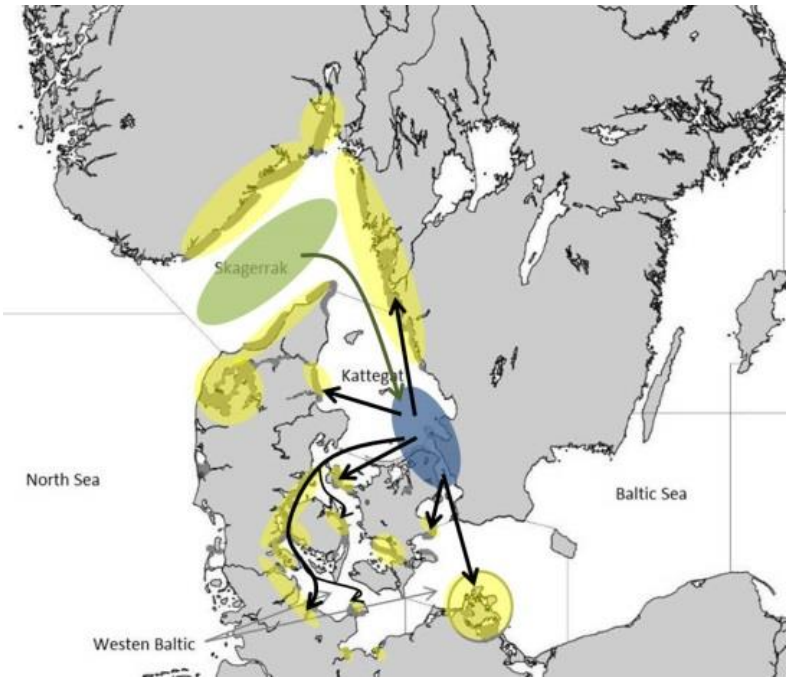


Fig. 2 Migration pattern of western Baltic spring spawning (WBSS) *Clupea harengus.* In summer, herring forms aggregation of mixed populations for feeding in the Skagerrak (green area). Overwintering takes place in the Sound (blue area), and during spring population segregate to different spawning grounds (yellow areas). Map modified from Clausen et al. (2015).

With those questions I formulated six hypotheses:

Hypothesis 1: Herring are locally adapted to the salinity at spawning grounds. Within population crosses show a better performance in their own salinity than the foreign within population cross.

Hypothesis 2: Between populations crosses show an intermediate performance compared to within population crosses, as the pattern of local adaptation is disrupted genetically.

Hypothesis 3: All crosses perform worst at a novel salinity not occurring in their natural spawning grounds.

Hypothesis 4: Exposed to a bacterial stress, offspring from within population crosses of parents spawning in higher salinity show a reduced performance and immune response compared to offspring from parents spawning in low salinity.

Hypothesis 5: Between population crosses show an intermediate pattern or even a better immune response than within crosses, because offspring show a diversified immune competence, as their parents transfer information from different immune histories (different habitats= different pathogen exposure).

Hypothesis 6: Offspring from populations of low saline water are better adapted to a *Vibrio* spp. stress and should therefore be better in coping with a combination of the two stressors (salinity and pathogen) than offspring from populations of higher saline waters.

3 Material and Methods

3.1 Experimental design

Two spawning areas of spring-spawning herring in the Baltic Sea were selected as sampling areas. The spawning area in the Kiel Canal (Rade, N 54°20.368'/E 9°44.965') is characterized by a lower salinity level of approximately 7 PSU, whereas the spawning area in the Little Belt (Skaerbaek, Denmark N 55°30.781'/E 9°37.598') shows a higher salinity level of approximately 20 PSU. The adult herring used in this experiment were caught by local fishermen's in mid-April 2015. At both locations eight females and eight males were sampled, adding up to a total of 32 herrings. Ripe individuals were recognized by their "running" condition, i.e. when eggs and sperms drop out of the cloaca, by none or a slight squeezing of the abdomen. The gametes were stripped post-mortem and directly at the corresponding locations. The milt was collected in plastic beaker and the sticky eggs spread on plastic slides (11x5 cm) ideally in two rows of one layer thick. Gametes were stored dry (undiluted) at 4 °C on ice as suggested by Blaxter (1955). Due to logistical reasons the whole sampling was performed on two consecutive days, starting in Denmark the first day and continuing at the Kiel Canal the next day. This implies that the Danish gametes were about 24 hours older than the Kiel Canal gametes. In a climate chamber at GEOMAR the gametes were crossed between and within both locations in a full-factorial design, resulting in four different crosses (Fig. 3). From here on the four crosses will be named as follow: KfKm, KfDm, DfKm, DfDm (K= Kiel, D= Danish, f= female, m= male). I exposed the fertilized eggs of each cross to three different salinity levels, 7 and 20 PSU correspond to the two study locations and 28 PSU served as a novel salinity. The procedure of fertilization was conducted as follow: The sperm were activated by pouring seawater into the beaker and slightly slewing for a few seconds. Sperm were activated in the salinity level of the males origin (Danish male at 20 PSU, Kiel Canal male at 7 PSU) to ensure an optimal activation. Always two slides with unfertilized eggs were put in tanks with seawater of the respective rearing salinity (7, 20 and 28 PSU). Fertilization was achieved by simply pouring the sperm solution into the tank. After 10 min fertilization was expected to be completed (Rosenthal, 1988) and the slides were put for another 10 min in a disinfection bath containing an Actomar solution (20mL Actomar/1L saltwater) to minimize the risk of fungal infection. Each tank was replicated eight times, resulting in 96 tanks (12x8 replicates) and so one tank represents one replicate (two slides). The climate chamber was kept for the whole experiment at 8 °C and low light. Light was regulated by a clock timer reflecting the local natural conditions (13h: 11h, light: dark). According to Peck (2012) western Baltic herring need 120 day degrees until 50% of the larvae have hatched, which corresponds to 15 days at 8 °C. The rearing tanks (19x11x13 cm) were painted dark green from the outside to prevent the larvae swimming along the walls. The tanks were filled with 1.5 L saltwater and a 50% water exchange was done daily, as no flow-through system was installed (Blaxter and Hempel 1961). To achieve the three salinity level I used UV treated North Sea water at 28 PSU and diluted it with tap water to 20 and 7 PSU.

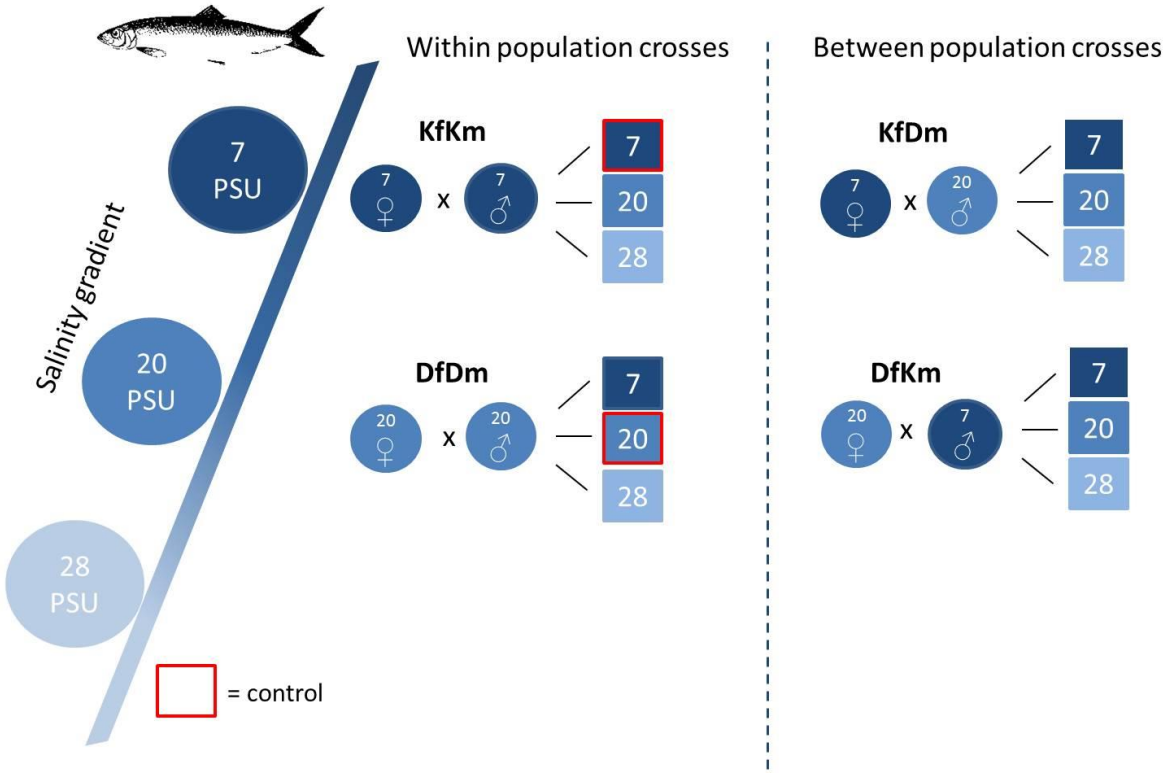


Fig. 3 Experimental design. All four different crosses are exposed to three salinity levels (7, 20 and 28 PSU). Abbreviations: K= Kiel, D= Danish, f= female, m= male

As a first life-history trait, I estimated the fertilization rate in each tank at day one and two post-fertilization. I counted the amount of fertile eggs and estimated the proportion of fertile eggs to the total amount of eggs. Fertilized eggs can be distinguished from unfertilized eggs under the binocular microscope, as fertilized eggs become more translucent and the formation of a perivitelline space becomes visible (Fig. 4).

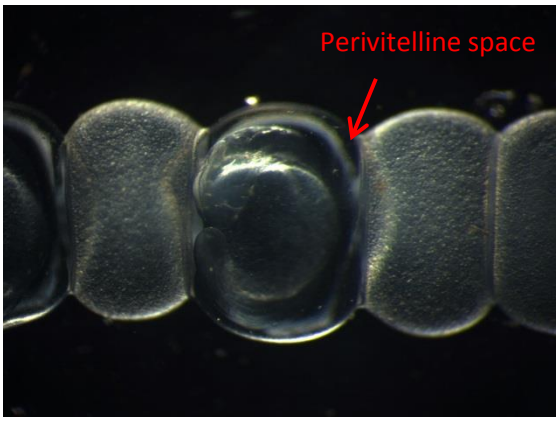


Fig. 4 Unfertilized eggs appeare here opaque white, the fertilized egg exhibits a perivitelline space between membrane and oocyte (here a 6 day old larvae).

The second life-history trait, hatching timing, was measured by recording the hatching peak in days post-fertilization of each tank. This time point will from now on be perceived as hatching time. Furthermore, all hatched larvae were counted to calculate hatching success. Hatching success in % was calculated as follow:

Hatched larvae * 100 / number of fertile eggs

Four days after the particular hatching peaks, a bacterial treatment (*Vibrio spp*.) was conducted with a subset of the larvae from each tank. The treatment was set at a time point were the larvae were still in the yolk-sack stage, so did not yet switch to exogenous feeding but already developed a mouth gap. The timing of mouth opening is important, because one possible entry for *Vibrio spp.* is via the mouth (other possible entries are also gills and anus Laurencin et al. 1987). From each tank

forty larvae were transferred and divided into two plastic beakers (6 dl) filled with the corresponding salinity level (20 larvae per beaker). One beaker served as a control and in the second beaker *Vibrio spp.* was added. The *Vibrio spp*. treatment was conducted with a foreign strain that was isolated in a previous study of Roth et al. from pipefish *Syngnathus typhle* occurring in Italy (strain I9K1). The *Vibrio spp.* strain was taken from a -80 °C glycerol stock, suspended in a liquid medium (Medium 101: 5 g peptone + 3 g meat extract + 30 g NaCl in 1 L Millipore-H_2O, autoclaved and kept at room temperature) and grown in an overnight culture at 25 °C. Thereafter, the bacteria solution was centrifuged for 10 min at 2'500 rpm and the supernatant removed. The bacteria pellet was then resuspended in autoclaved seawater (7, 20 and 28 PSU) according to the salinity treatment in the beakers, yielding a concentration of 10^9 bacteria cells mL^{-1}. Finally 5 mL of the bacteria suspension was added to the *Vibrio* treatment beaker generating a concentration of 10^7 cells mL^{-1}, respectively 5 mL of autoclaved seawater was added to the control beakers. 24h after the *Vibrio* treatment, 15 larvae from each beaker were sampled for gene expression analysis. Larvae were put directly in a RNA stabilizing reagent on ice (RNAlater®). Samples were then kept one day at 4 °C and then stored at -20 °C for further analysis.

The five leftover larvae per beaker were used for survival analysis. Mortality was recorded daily until the last larvae died. In the tanks mortality was checked daily as well and the dead larvae were removed. This created two survival analysis datasets: one dataset with a controlled amount of larvae and *Vibrio spp.* as an additional stressor (beaker dataset), and a second dataset with different larvae density and only salinity as a stressor (tank dataset).

3.2 Genes of interest

For the gene expression analysis a set of 32 genes was analyzed and the genes were grouped according to their functions (Table 1). Except from the epigenetic genes, all other genes (primers) were taken from the master thesis on Atlantic herring of Luisa Listmann (2014).

Table 1 Genes analysed for their expression profile. Genes are sorted in six groups according their functions in the organism.

Gene group	Gene name	Abbreviation	Function	Source
Epigenetic gene	Histone-acetyltransferase KAT2A (Bromodomain)	BROMO	Acetylation of histones at lysine residues (gene activation)	NCBI
	Histone-acetyltransferase KAT2A (PCAFdomain)	PCAF	Acetylation of histones, predominantly helical (gene activation)	NCBI
	Histone-acetyltransferase KAT8 (MOZ/SASdomain)	MOZ	Acetylation of histones (gene activation)	NCBI
	Histone-deacetylase 1B (HDAC1domain)	HDAC1	Deacetylation of histone at lysine residues, involved in DNA-damage response (gene silencing)	NCBI, UniProt
	Histone-methyltransferase (SPRYdomain Ash2)	SPRY	Methylation of histones, Ash2 protein involved in transcriptional regulators of Hox genes (gene silencing)	NCBI, UniProt

	Lysine-specific demethylase_6A (TPRdomain)	TPR	Demethylation of histones (gene activation)	NCBI
	DNA(cytosine-5)-methyltransferase1 (RFDdomain)	RFD	Methylation of cytosine, involved in DNA repair and genome stability (gene silencing)	NCBI
	DNA(cytosine-5)-methyltransferase3A (ADDZdoamin)	ADDZ	Methylation of cytosine, inheritance of pattern during mitosis (gene silencing)	NCBI
Houskeeping gene	18s ribosomal RNA	18s rRNA	Protein synthesis	NCBI
	β-actin	beta-actin	Cell motility, cytoskeleton	NCBI
	Elongation factor 1α	EF1-alpha	Involved in deliver of tRNAs to ribosome	NCBI
Immune genes	Gene associated with retinoic-interferon-induced mortality 19	GRIM19	Innate immunity, cell death regulation	NCBI
	Natural resistance associated macrophage protein	NRAMP	Innate immunity, metal transport in particular iron	UniProt
	IK cytokine	IK Cytokine	Adaptive and innate immunity, signalling protein between humoral and cell-based immune response	Foster 2001
	Kinesin family member 13b	KFM 13b	Innate immunity, immune involvement?	
	Integrin β1	Integrin-beta 1	Adaptive immunity; cell adhesion	NCBI
	Natural killer enhancing factor	NKEF	Innate immunity	
	Akirin	Akr	Innate immunity, downstream effector of the Toll-like receptor	UniProt
	Tumor necrosis factor 2	TNF2	Innate immunity, inflammation response	UniProt
	Translocator protein	TSPO	Innate immunity , inflammation response, immunomodulation	
	Complement component 1 Q subcomponent-binding	CC1Qsub	Innate immunity, complement system	UniProt
	Complement component C3	CC3	Innate immunity, complement system	UniProt
Osmoregulation gene	Na^+-K^+-ATPase	ATN-A1 I	Osmoregulation, ion exchange	Varsamos 2010
	V-type-H^+- ATPase subunit A	V-H-ATPase	Osmoregulation, ion exchange	Varsamos 2010
	Na^+/H^+ exchanger 1	NHE1	Maintaining of ion homeostasis	Vilella 1995
	Na^+/HCO_3^+ cotransporter	NBC1	Osmoregulation	Taylor 2010
Stress	Heat shock protein 70	hsp70	Stress-induced response,	UniProt

gene			chaperone for unfolded protein	
	Heat shock factor 1	hsf1	Stress-induced response, DNA-binding protein	UniProt
	Growth arrest and DNA damage inducible protein 45α	GADDIP45a	Activated by DNA damage	UniProt
	Heat shock protein DNAj4	hspDnaj4	Stress-induced response, chaperone for unfolded protein	UniProt
	Heat shock protein 90	hsp90	Stress-induced response, chaperone for unfolded protein	UniProt
Metabolism gene	Apolipoprotein E	Apolip E	Lipid metabolism	UniProt

3.3 RNA extraction and cDNA synthesis

The frozen larvae in RNAlater® were thawed on ice and transferred to 750 µL QIAzol lysing reagent (Qiagen) and kept at -20 °C. Always three full-sib larvae were pooled to ensure enough raw material for the further gene expression analysis. The next day, the plates were again thawed on ice. To ensure optimal tissue lysis, ceramic beads (1x2.8 mm, 2x1.4 mm) were added and the plates shaken twice for 5 min at 25 Hz. The RNA extraction was processed following the protocol of the RNAeasy 96 Kit (Qiagen). After lysis, 150 µL chloroform was added to separate the lysate in two phases, where the upper aqueous phase contains RNA and was then transferred to a new plate. After adding 400 µL of 70 % ethanol the whole lysate was transferred to a RNA binding-column and centrifuged for 4 min at 6000 rpm. RNA was washed in three steps and then eluted in 45 µL H_2O, the concentration of RNA was measured using Nanodrop.

For the cDNA synthesis the QuantiTect® Reverse transcription kit (Qiagen) was used. RNA and reagents were thawed on ice. First a genomic DNA elimination reaction was performed to ensure that there is no mixing of cDNA and gDNA at the end of the synthesis, as only the transcribed information is of interest for gene expression analysis. To achieve the same RNA concentration among all samples a RNA template was performed, taking 250 ng RNA and adding H_2O to a final volume of 7 µL. To the 7 µL RNA template 1 µL gDNA wipeout buffer was added and then incubated for 2 min at 42 °C. In a second step the RNA was reverse transcribed by taking 7 µL RNA (from the gDNA wipeout reaction), adding 0.5 µL reverse-transcription master mix, 2 µL Quantiscript RT Buffer, 0.5 µL RT Primer mix and incubate for 15 min at 42 °C and 3 min at 95 °C. Finally 25 ng μL^{-1} cDNA were obtained and stored at -80 °C. The 1 µL per sample left over from the gDNA elimination reaction were pooled and used later as a control (-RT control).

3.4 Primer design and primer testing

In total 32 target genes were analysed, where from 24 genes (11 immune genes, 5 stress genes, 4 osmoregulation genes, 3 housekeeping genes, 1 metabolism gene) primers designed by Luisa Listmann in the framework of her master thesis (2014) could be used. In addition, eight primers for epigenetic genes were designed, in which four epigenetic genes were involved in gene silencing and four in gene activation. The herring transcriptome provided by Lamichhaney et al. (2012) was used to find candidate genes. First the transcriptome was transformed to a blast-database using the

software BLAST (NCBI). Sequences (query) from epigenetic genes of the pipefish *Syngnathus typhle* (provided by Anne Beemelmanns) were blasted against the herring transcriptome to find matching sequences. Conserved regions and domains with functions were searched using Blastx and Blastn (NCBI) in the output sequences, this defined sequences were the basis for primer design. Primers were designed using the software Primer3 and the NCBI Primer designing tool. The transcriptome was used as a reference database to avoid multiple amplifications. The primer parameters were determined as follow: melting temperature 59-60 °C, amplicon length 80-200 bp, primer length 20-24 bp, 40-60 % GC-content; max. 3' complementary score of 4-5; max. of 3 di-nucleotide repeats; max. 4 bp runs. The proposed primer pairs were checked by eye for primers avoiding GC-clamps, repetition of G's and C's and interactions of forward and reverse primers.

Primer quality and efficiency was tested using real time quantitative PCR (RT-qPCR; StepOnePlus™ Cycler, Applied Biosciences). For this purpose, 1 µg RNA (of three surplus larvae) was reversed transcribed. With the obtained cDNA, a 6 step dilution series (1:10, 1:30, 1:90, 1:270, 1:810 and 1:2430) was established serving then as template. For the qPCR each well contained 2 µL 5xHot FIREPol® EvaGreen® (Solis BioDyne), 0.5 µL forward and reverse primer (diluted 1:10 to 10 pmol mL^{-1}), 5 µL H_2O , 2 µL template and each reaction was performed as triplicate. The RT-qPCR run was performed with the following protocol: an initial phase of 15 min at 95 °C, 45 cycles of 15 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C, a melt curve stage of 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C.

Primers with an efficacy range of 90-100 % and a R^2 value of >0.9 were used for gene expression measurements. Four primers showed an efficacy over 100 %, I decided to include them as well in the analysis because their R^2 value was over 0.9 and the amplification and melting curves looked acceptable (see appendix: Table 8.1).

3.5 Gene expression assay

The Fluidigm Dynamic Array™ IFC Chip has 96 inlets for each sample and primer assays. To allow for a statistical analysis, measurements are performed in technical triplicates. This implies that 32 primer pairs could be analysed on 96 samples. Gene expression assay was performed following the Fluidigm 96/96 protocol. In a first step a pre-amplification (preAmp) of the target cDNA was done to ascertain that sufficient material for the fluidigm RT-qPCR was available. One PreAmp-reaction contained 2.5 µL TaqMan PreAmp MasterMix (Applied Bioscience®), 0.5 µL STA Primer mix (containing all primer pairs 50 µM diluted in low EDTA TE Buffer). The amplification was run under the following conditions: activation phase of 10 min at 95 °C, 16 cycles of 15 s at 95 °C and 4 min at 60 °C. Subsequently, the preAmp was diluted 1:20 with EDTA TE buffer and 16 randomly selected samples were checked with RT-qPCR if amplification was successful. The preAmp plates were stored at -20 °C. For the Fluidigm Chip run a "primer plate" and a "sample plate" were prepared. The primer plate contained 0.7 µL PrimerPreMix (50 µM primer pair), 3.5 µL 2xAssay loading reagent (Fluidigm) and 3.15 µL 1xlow EDTA TE buffer. The sample plate contained 3.3 µL preAmp cDNA, 3.5 µL 2xSSoFast EvaGreen Supermix with low Rox (BioRAD) and 0.35 µL 20xDNA Binding Dye Sample loading reagent (Fluidigm). After priming the chip with control line fluid, 5 µL sample and 5 µL primer assays were loaded on the chip and bubbles removed with a one-way injection cannula. As

negative controls water samples as well as –RT samples were included. To control for technical bias samples were distributed randomly over the chips.

3.6 Statistical analysis

All statistical analyses were performed in RStudio (R core team, 2015). The first part of the life-history data (fertilization rate, hatching rate and timing) were checked for normality using Shapiro-Wilk test and heterogeneity of variances using Levene's test. A two-way ANOVA was performed to analyse main effects and interaction of the factors salinity level and crossing type. A post hoc test (Tukey HSD test) elucidated the significant differences between factor levels. The survival data contained no censored data and were analysed with either a two- or a three-way ANOVA with a subsequent post hoc test (Tukey HSD test) to find the differences among the single survival curves.

The raw gene expression data were accessed via the Fluidigm real-time PCR analysis software (Fluidigm) to evaluate the amplification profiles and exclude not or bad amplified samples. For the technical triplicates mean, standard deviation (SD) and the coefficient of variation (CV= SD/mean) were calculated. CV gives an indication how precise a measurement is and if CV is >0.04 the value is falsified by a measurement error. Missing data points (2.4% of total data) and data points of CV>0.4 (0.1 % of total data) were replaced with the mean value over all samples of the respective gene. The most stable gene combination over all samples was analysed using the qbase+ software (Biogazelle). With the geNorm algorithm reference genes (housekeeping genes) are found among all candidate genes. Based on these references genes, a gene expression normalization factor can be calculated by the geometric mean of those housekeeping genes. Thereby it is possible to calculate the relative expression value (δCt) of each sample. In all graphs and analysis the – δCt (geometric mean of reference genes minus target gene) is shown. This simplifies the interpretation as positive values mean an upregulation and negative values a downregulation of the target gene relative to the housekeeping genes. Gene expression data were checked for normality using Shapiro- Wilk test and heterogeneity of variances using Levene's test. With an adjusted quantile plot multivariate outliers were detected (Filzmoser et al. 2014). In this approach the cut-off value for detecting outliers is defined by the Mahalanobis distance (multidimensional measure of how many SD a point is away from the mean). More precisely, if a data point falls outside the 97.5% quantile of a Chi-square distribution it is identified as an outlier. In this case, the six most extreme samples were deleted from further analysis. First a multivariate approach using PERMANOVA was applied to the whole dataset and to the different gene groups to evaluate main effects and interactions of the three factors (salinity level, cross type and *Vibrio* treatment). For better visualization principal component analysis (PCA) plots were performed. Main effects and interactions on single genes were analysed with a three-way ANOVA and type "III" sums of square corrected for unbalanced design. To elucidate the significant differences between factor levels a Post hoc test (Tukey HSD test) was applied.

4 Results

4.1 Life-history traits

Fertilization rate - For analysing fertilization rate 6-8 replicates per treatment group were included. In seven tanks fertilization failed due to two Danish females with bad quality eggs (partially bloody). The ANOVA yielded a significant main effect of crossing ($p < 0.05$*) and salinity ($p < 0.001$***) on fertilization rate (Table 2). The post hoc test showed that fertilization rate was highest in the lowest salinity level (7 PSU), followed by 20 PSU and was lowest in 28 PSU (Fig. 5).

Table 2 ANOVA and Tukey post hoc test of salinity and crossing effect on fertilization rate**.** Abbreviations**:** K= Kiel, D= Danish, f= female, m= male * denotes a significant result (p<0.05), . denotes a trend (p<0.1) and ns a not significant result.

ANOVA	df	F value	P
Crossing	3	3.224	**0.0271***
Salinity	2	41.754	**5.23e-13 *** **
Crossing*Salinity	6	2.10	0.0627 .
Tukey post hoc			
7PSU- 20 PSU			**0.036***
7 PSU- 28 PSU			**0.00*** **
20 PSU- 28 PSU			**0.00*** **
DfKm- DfDm			0.8917 ns
KfDm-DfDm			0.2995 ns
KfKm-DfDm			0.3769 ns
KfDm-DfKm			0.0689 .
KfKm-DfKm			0.0953 .
KfKm-KfDm			0.9983 ns

Hence, fertilization rate decreased with increasing salinity. For that main effect of crossing, the posthoc test revealed trends, where KfDm and KfKm tended to have a higher fertilization rate than DfKm (Fig. 6)

The main effect of crossing on fertilization could not be verified by the post hoc test, nevertheless two trends indicate that both crosses with a Kiel female (KfDm and KfKm) tended to have a higher fertilization rate than the between population cross DfKm (Fig. 6). The main effect of salinity was not dependent on crossing, however the ANOVA yielded a trend on an interaction of both factors (Table 2) and this trend seem to be among both within population crosses (Fig. 7). If only both within population crosses (KfKm and DfDm) are considered in the analysis a significant interaction between crossing and salinity can be detected ($p = 0.00934$ **, df= 2, F value= 5.280). Thereby, in 7 PSU KfKm has a higher fertilization rate than DfDm, but in 20 PSU and 28 PSU this difference is vanished (see appendix: Table 8.2).

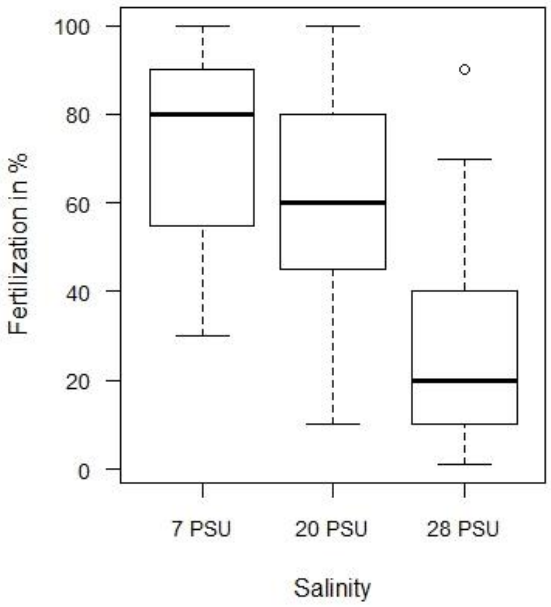


Fig. 5 Effect of salinity on fertilization rate (box-whisker plot).

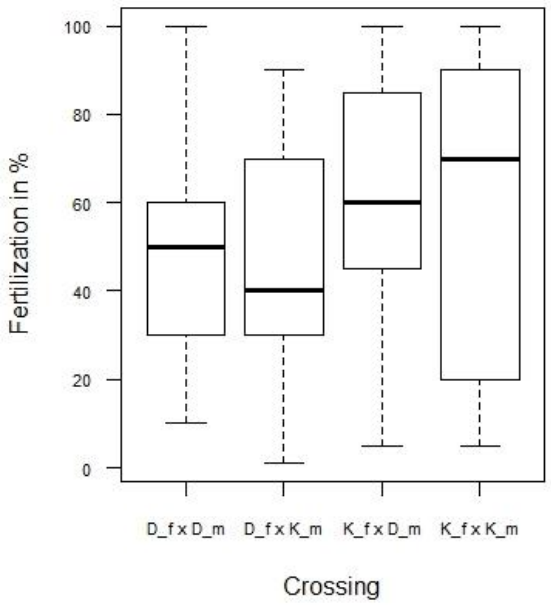


Fig. 6 Effect of crossing on fertilization rate (box-whisker plot)

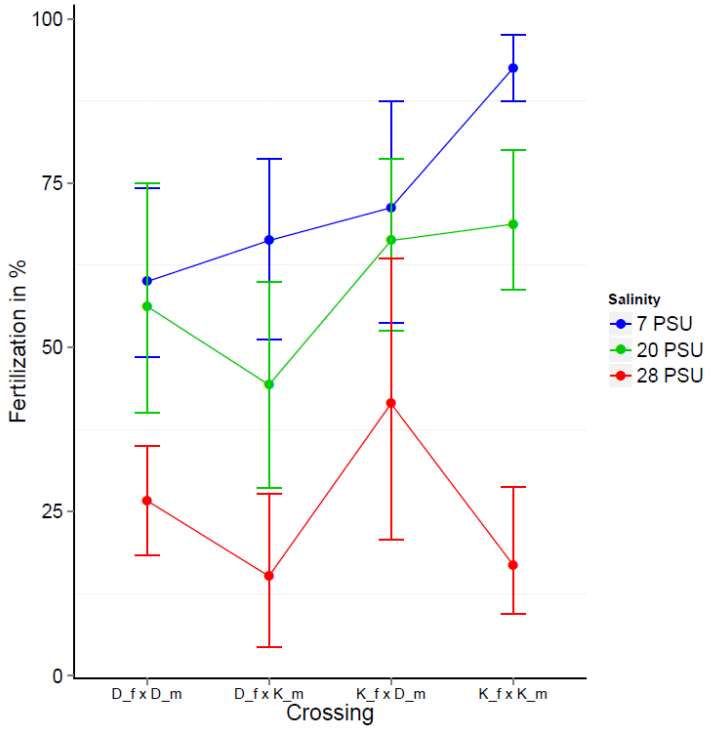


Fig. 7 Interaction plot of crossing and salinity on fertilization rate. Mean and 95% confidence interval are shown

Hatching time- For the hatching time 6-8 replicates per treatment group were included in the analysis, but from one treatment group (DfKm in 28 PSU) only 3 replicates remained. The other tanks had to be excluded due to a total loss of the eggs. The ANOVA yielded a significant main effect of crossing ($p < 0.05$*) and salinity ($p < 0.001$***) on hatching timing but no effect on the interaction of both factors (Table 3). The multiple comparisons of the factor levels (post hoc test) revealed significant differences among all three salinity levels and a significant difference between the crosses DfKm vs. DfDm and KfKm, as well as a trend to KfDm. Figure 8 indicates that the larvae of the cross KfDm hatched earlier than the other three crosses.

Table 3 ANOVA and Tukey post hoc test of salinity and crossing effect on hatching timing**.** Abbreviations**:** K= Kiel, D= Danish, f= female, m= male
* denotes a significant result (p<0.05), . denotes a trend (p<0.1) and ns a not significant result.

ANOVA	df	F value	P
Crossing	3	3.727	**0.0152 ***
Salinity	2	60.998	**5.56e-16 *****
Crossing*Salinity	6	1.370	0.2393 ns
Tukey post hoc			
7PSU- 20 PSU			**2.00e-07*****
7 PSU- 28 PSU			**0.00*****
20 PSU- 28 PSU			**1.47e-05*****
DfKm- DfDm			**0.0380***
KfDm-DfDm			0.9939 ns
KfKm-DfDm			0.9954 ns
KfDm-DfKm			0.0606 .
KfKm-DfKm			**0.0179***
KfKm-KfDm			0.9576 ns

On average larvae reared in 7 PSU hatched after 17.6 days post-fertilization (dpf; Table 4). Two days later those larvae reared in 20 PSU hatched (mean 19.4 dpf) and another two days later larvae reared in 28 PSU hatched (mean 21.3 dpf). Hatching timing was prolonged with increasing salinity in a mean interval of two days.

Table 4 Mean, minimum and maximum day post-fertilization (dpf) on the different salinity level.

Salinity	mean dpf	min dpf	max dpf
7 PSU	17.6	15	20
20 PSU	19.4	18	22
28 PSU	21.3	18	23

In Fig. 9 it is shown that the main effect salinity was not dependent on crossing. If only the within population crosses (KfKm and DfDm) are considered in the ANOVA no effect but a trend (p= 0.0839, df= 2, F value= 2.656) can be detected for the interaction of salinity and crossing on hatching timing.

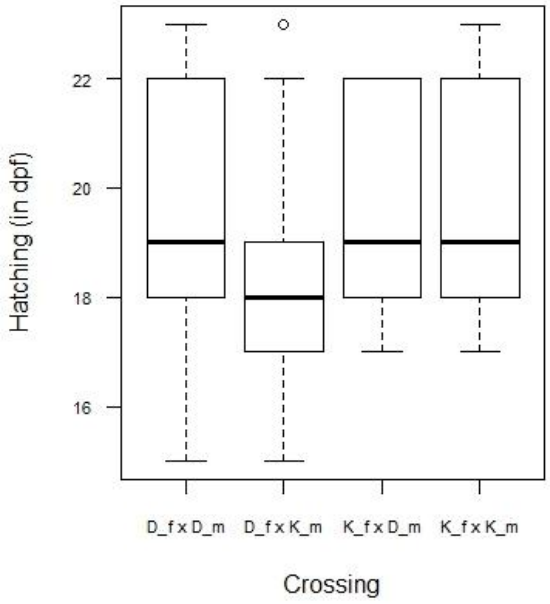


Fig. 8 Effect of crossing on hatching timing (box-whisker plot)

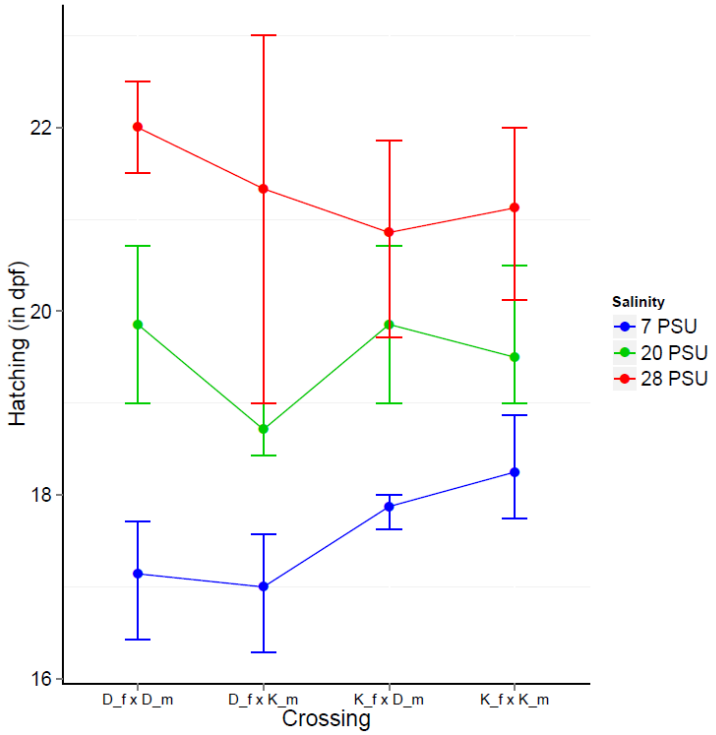


Fig. 9 Interaction plot of crossing and salinity on hatching timing. Hatching in days post-fertilization (dpf). Mean and 95% confidence interval are shown.

Hatching rate- For the hatching rate 6-8 replicates per treatment group were included, seven tanks with total loss of eggs were defined as hatching rate of zero. Three out of these seven tanks were from the DfKm cross in 28 PSU and one tank in 7 PSU. The ANOVA indicates a significant main effect of crossing ($p < 0.001$***) on hatching rate, but no main effect of salinity and the interaction of both factors (Table 5). Tukey post hoc test revealed significant differences between all crossing levels, except for DfKm vs. DfDm and KfDm vs. KfKm.

Table 5 ANOVA and Tukey post hoc test of salinity and crossing effect on hatching rate**.** Abbreviations: K= Kiel, D= Danish, f= female, m= male
* denotes a significant result ($p<0.05$) and ns a not significant result.

ANOVA	df	F value	P
Crossing	3	7.492	**0.000183 *** **
Salinity	2	0.439	0.6465
Crossing*Salinity	6	1.168	0.3323
Tukey post hoc			
DfKm- DfDm			0.8919 ns
KfDm-DfDm			**0.0019****
KfKm-DfDm			**0.0026****
KfDm-DfKm			**0.0181***
KfKm-DfKm			**0.0243***
KfKm-KfDm			0.9990 ns

The hatching rate was higher for both crosses including a Kiel female compared to both crosses including a Danish female, implying that the main effect of crossing on hatching rate is driven by a maternal component (Fig. 10).

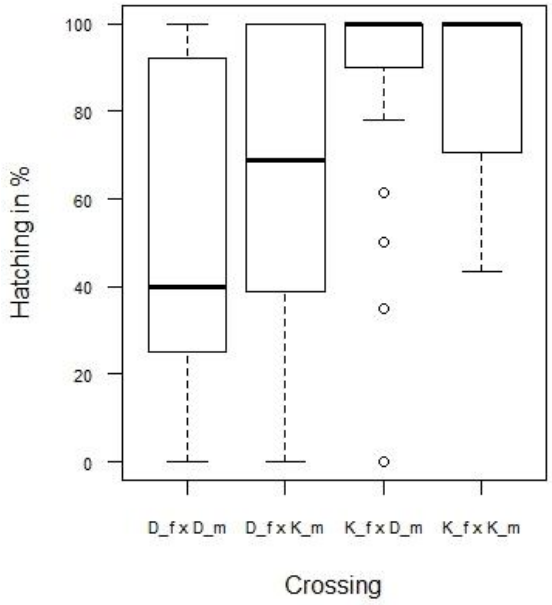


Fig. 10 Effect of crossing on hatching rate in % (box-whisker plot)

Mortality tank dataset- As mortality was measured until the last larvae died, the data contained no censored data. ANOVA indicated a main effect of salinity ($p < 0.001$***) on survival as well as a significant interaction of salinity and crossing (Table 6).

Table 6 Survival analysis (tank dataset) using ANOVA on salinity and crossing effect

ANOVA	df	F-value	P
Crossing	3	2.512	0.0568 .
Salinity	2	187.81	**< 2e-16 *** **
Crossing*Salinity	6	10.843	**5.09e-12 *** **

The post hoc test revealed significant differences among all salinity level (see appendix: Table 8.3), meaning that larvae reared in lowest salinity had the highest survival rate, followed by 20 PSU and 28 PSU. Fig. 11 shows the interaction of crossing and salinity sorted by the four different crosses. KfKm and KfDm show best survival in 7 PSU and worst survival in 28 PSU (all salinity levels differ significantly). However, for the DfDm cross the survival curves of larvae in 7 and 20 PSU do not differ significant, implying that in both salinity levels survival is better than in 28 PSU. Figure 12 shows the same data but sorted by salinity level. In 7 PSU the KfDm and KfKm crosses have a better survival in the first days than the DfDm and DfKm crosses. However, this effect vanished in 20 PSU, where only DfDm has a better survival than KfKm (survival curve of DfDm slightly shifted to the right). In 28 PSU both between population crosses showed a significantly better survival than the within population crosses.

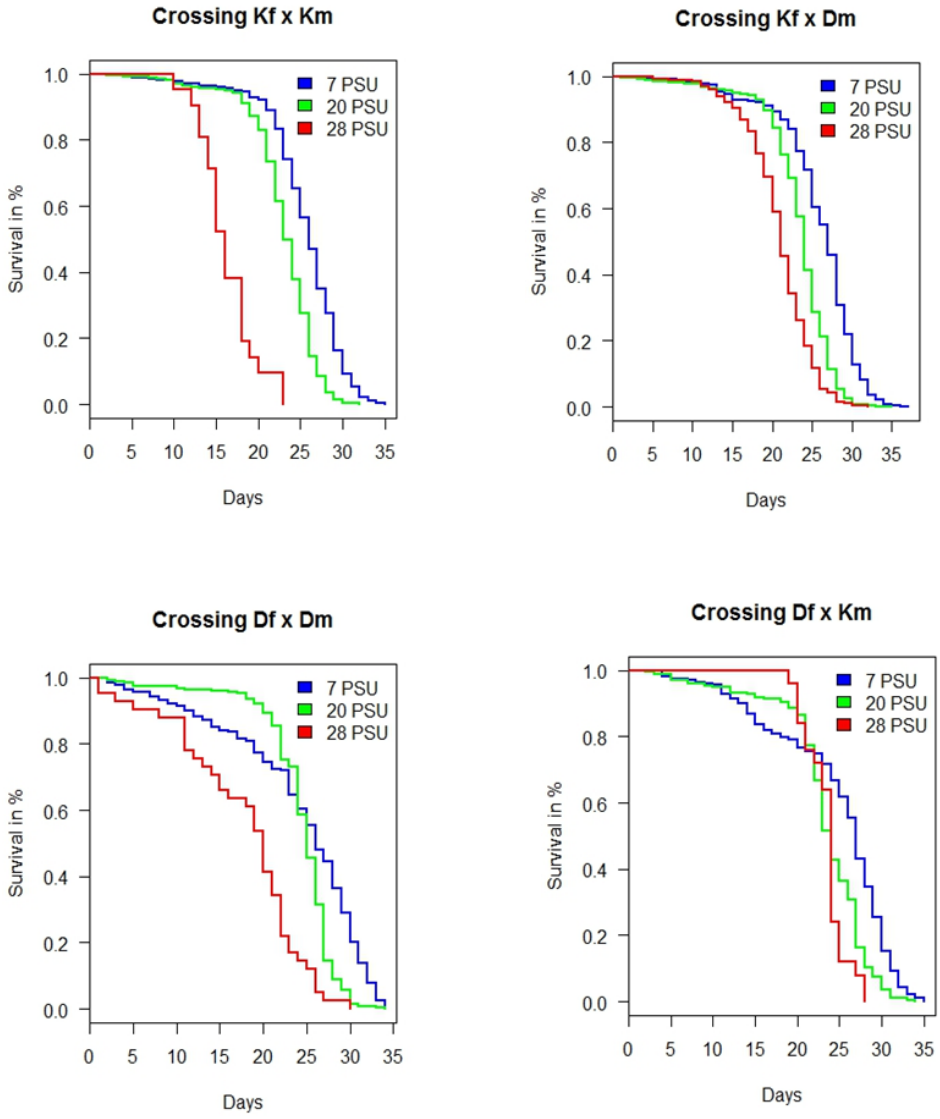


Fig. 11 Effect of salinity on survival for each cross. Survival in % (1.0 = 100%)

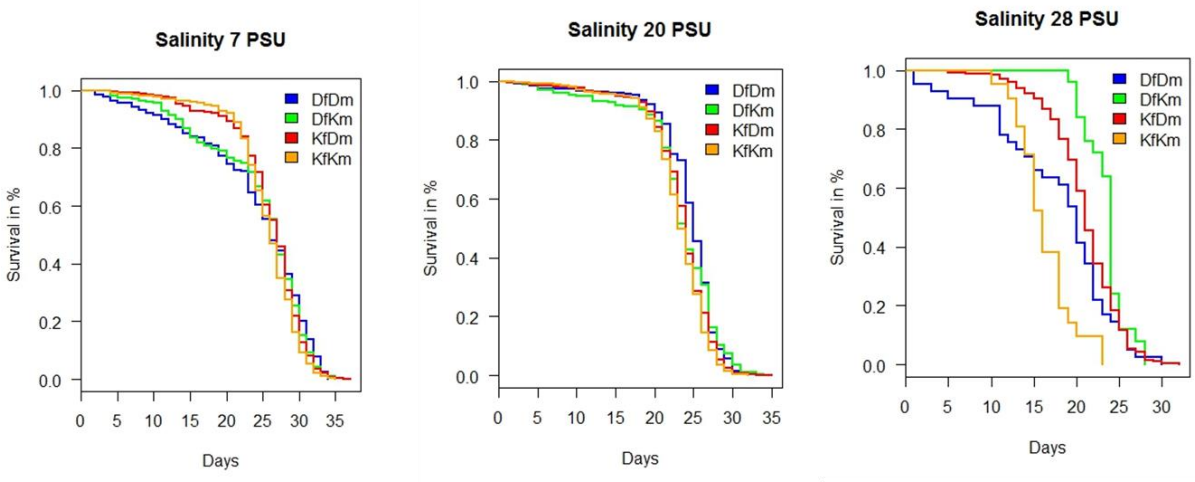


Fig. 12 Interaction of salinity and crossing on survival. Survival in % (1.0 = 100%)

Mortality beaker dataset- The mortality of all five larvae was measured, therefore also this dataset contained no censored data. The ANOVA output indicated a significant main effect of salinity (p <0.05*) and *Vibrio* (p <0.001***) as well as significant interactions of crossing*salinity (p <0.001***), *Vibrio*salinity (p <0.001***) and crossing**Vibrio*salinity (p <0.001***) (Table 7).

Table 7 Survival analysis (beaker dataset) using ANOVA on salinity, crossing and *Vibrio* effect

ANOVA	df	F-value	P
Crossing	3	2.081	0.1016
Salinity	2	3.180	**0.0423 ***
Vibrio	1	55.660	**3.06e-13 *****
Crossing*Salinity	6	4.044	**0.0005*****
Crossing*Vibrio	3	1.544	0.2019
Vibrio*Salinity	2	11.135	**1.79e-05 *****
Crossing*Salinity*Vibrio	6	4.161	**0.0004 *****

The post hoc test (see appendix: Table 8.4) elucidated no significant differences among the salinity level in the control treatment (Fig. 13). However, in the *Vibrio* treatment larvae in 28 PSU had a significant better survival than larvae in 7 and 20 PSU. Moreover, the survival of larvae in 28 PSU with *Vibrio* or control treatment did not differ. Though, for larvae in 7 and 20 PSU the survival was best in the control treatment (Fig. 13). The threefold interaction yielded a significant better survival for the KfKm cross than the DfDm cross in the control treatment of 7 PSU (p= 0.0012**) (Fig. 14).

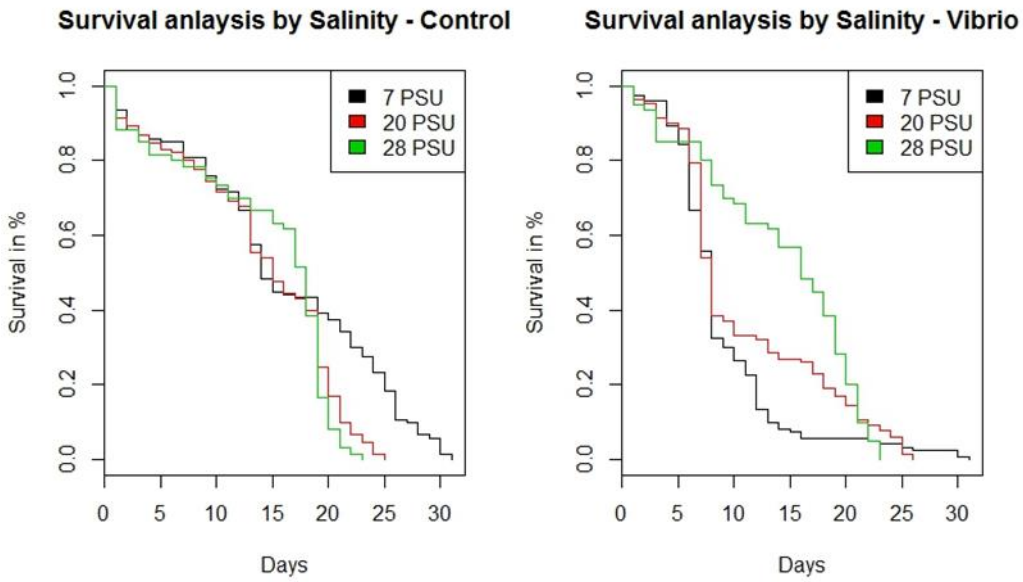


Fig. 13 Comparison of the salinity effect on survival in Vibrio and control treatment. Survival in % (1.0 = 100%)

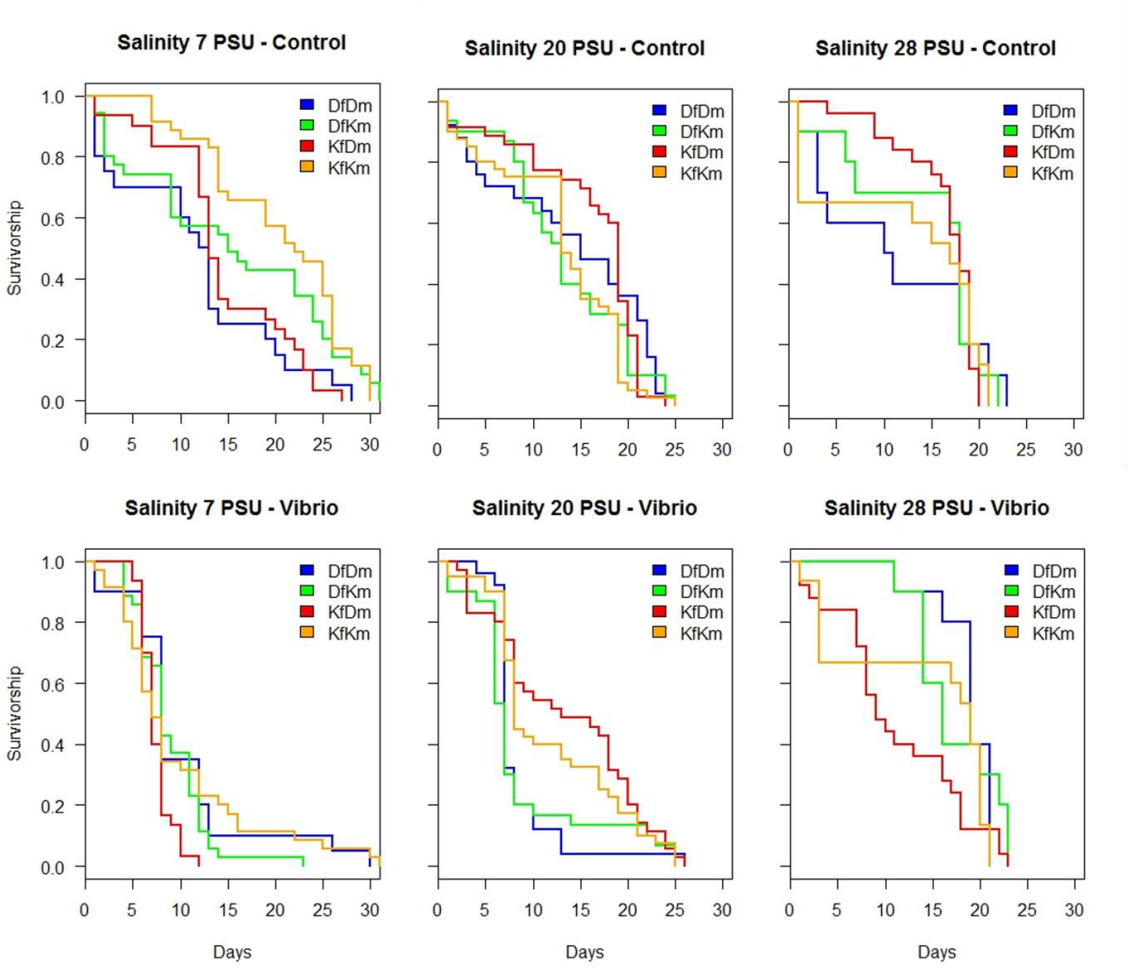


Fig. 14 Interaction of crossing and salinity on survival among Vibrio and control treatment. Survival in % (1.0 = 100%)

4.2 Gene expression

The most stable gene combination according the geNorm analysis, were the two epigenetic genes (silencing) RFD and SPRY. They will be called from now on "housekeeping genes". As the housekeeping genes differ from those of Listmann's master thesis, the genes 18sr RNA, β-actin and EF1α will from now on form the group "basic cell function genes". From each treatment group 3-7 datapoints could be included into the analysis (see appendix: Table 8.5) and six samples were detected as outlier and excluded from the analysis. As many samples from 28 PSU were missing, the analysis of gene expression was only done for 7 and 20 PSU. The PERMANOVA over the whole dataset yielded a significant main effect of crossing ($p < 0.05$*) on gene expression (Table 8). Salinity and *Vibrio* as well as all interactions showed no effect. The principal component analysis (PCA) showed a clustering of DfDm and DfKm, though KfKm and especially KfDm cluster apart from the Danish crosses (Fig. 15). The first principal component (PC) explains 44.12% and the second PC 13.5% of the variances.

Table 8 PERMANOVA of salinity, cross and *Vibrio* effect on gene expression in –δCt of all genes combined

PERMANOVA	df	R^2	P
Crossing	3	0.0774	**0.021 ***
Salinity	1	0.0097	0.508
Vibrio	1	0.0069	0.719
Crossing*Salinity	3	0.02511	0.807
Crossing*Vibrio	3	0.00984	1.000
Vibrio*Salinity	1	0.01654	0.224
Crossing*Salinity*Vibrio	3	0.03193	0.589

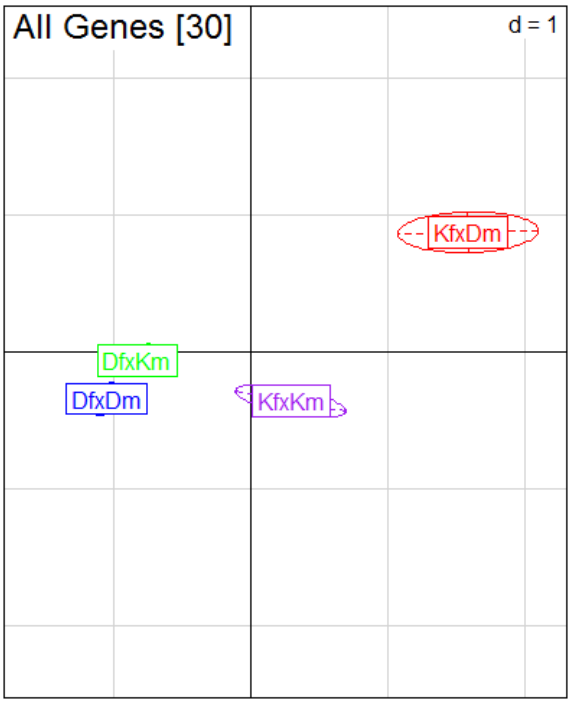


Fig. 15 Principal component analysis (PCA) on all genes plotted by crossing. The first principal component (x-axis) explain 44.12 % of the variance, the second principal component (y-axis) explains 13.5 % of the variances. Abbreviations: K= Kiel, D= Danish, f= female, m= male.

Furthermore, the PERMANOVA over different groups yielded significant main effects of crossing on "immune genes" ($p < 0.05$*) and "epigenetic genes- silencing" ($p < 0.05$*) as well as an interaction of *Vibrio**salinity on "epigenetic genes- activation" ($p < 0.05$*). The "Basic cell function genes" ($p=0.089$) and "osmoregulation genes" ($p=0.056$) showed a trend for a crossing impact on gene expression (see appendix: Table 8.6). The PCA showed for immune genes and epigenetic genes a similar clustering of crossing as for all genes combined (Fig. 16). For the interaction of salinity and *Vibrio* treatment the PCA indicated a grouping of both *Vibrio* treatment and the control 20 PSU but a divergent clustering when larvae were exposed to 7 PSU (control) (Fig. 16).

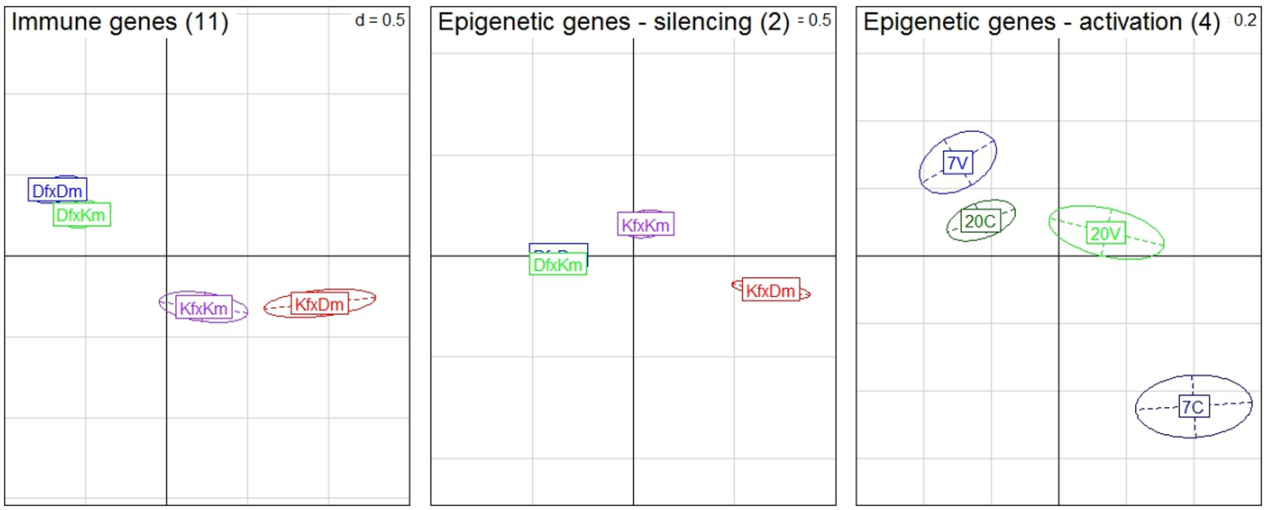


Fig. 16 Principal component analysis (PCA) from left to right: immune genes plotted by crossing, epigenetic genes (silencing) by crossing and epigenetic genes (activation) plotted by interaction of *Vibrio* and salinity. The first principal component (x-axis) explain 44.12 % of the variance, the second principal component (y-axis) explains 13.5 % of the variances. Abbreviations: K= Kiel, D= Danish, f= female, m= male, V= Vibrio, C= Control, 7= 7 PSU and 20= 20 PSU.

ANOVA of single genes yielded significant effects in five genes. A significant main effect of crossing was found in the immune gene CC3 ($p < 0.01^{**}$), the epigenetic gene HDAC1 ($p < 0.05^{*}$) and the stress gene hspDNAj4 ($p < 0.05^{*}$) (Fig. 17; see appendix: Table 8.7). The immune gene CC3 was in general more downregulated in crosses with a Danish female compared to crosses with a Kiel female. HDAC1 was significantly more downregulated in KfDm than in DfDm and DfKm (however consider the large standard error in KfDm). The stress gene hspDNAj4 was as well significantly downregulated in KfDm versus DfKm. A significant interaction of salinity*_Vibrio_ was found in the immune gene TNF2 ($p < 0.05^{*}$) and the epigenetic gene TPR ($p < 0.01^{**}$) (Fig. 18). The immune gene TNF2 showed in the control treatment no different expression pattern, but with *Vibrio* treatment TNF2 was downregulated in 20 PSU and upregulated in 7 PSU relative to the control treatment. The epigenetic gene TPR was in general downregulated in 20 PSU and upregulated in 7 PSU relative to the control treatment (see appendix: Table 8.8).

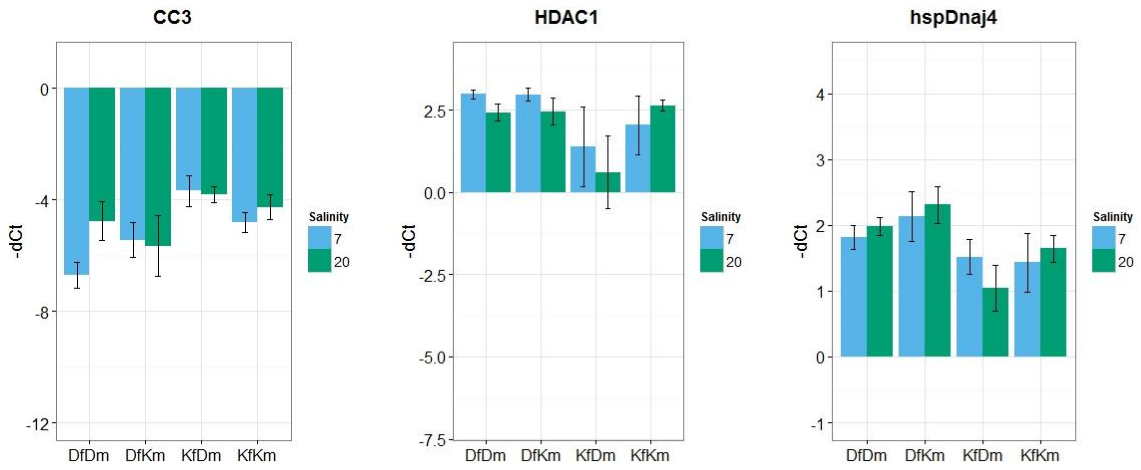


Fig. 17 Crossing effect on relative gene expression (–δCt). From left to right: immune gene, epigenetic gene and stress gene. Bar charts with standard error.

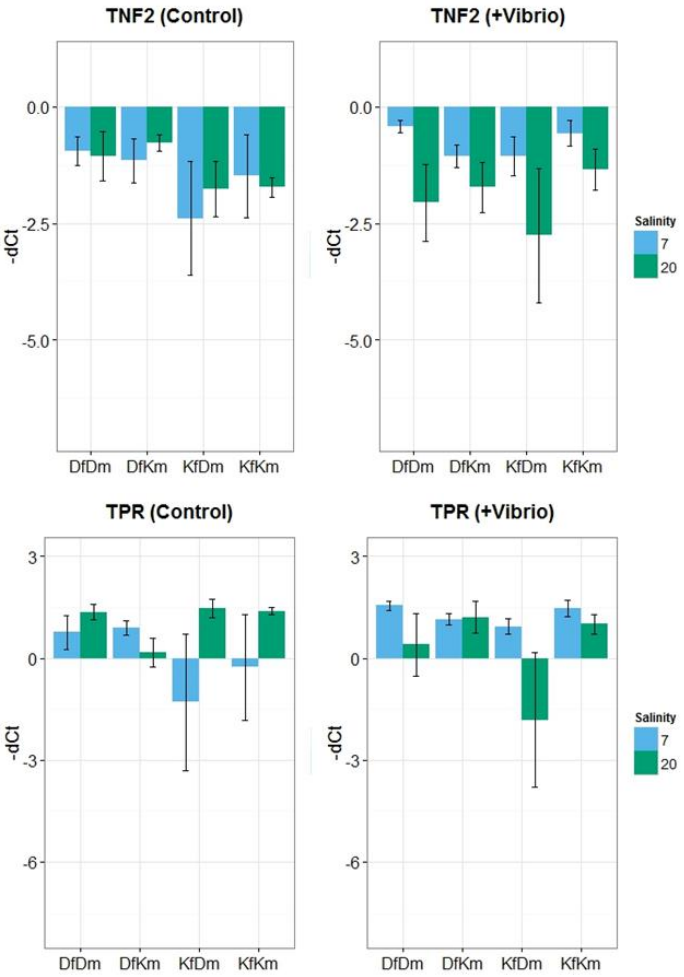


Fig. 18 Interaction of salinity and *Vibrio* on relative gene expression (–δCt). TPR (epigenetic gene) and TNF2 (immune gene). Bar charts with standard error.

5 Discussion

The assumption of local adaptation in western Baltic spring-spawning herring according to the salinity at spawning ground was until now solely based on neutral genetic markers (Bekkevold et al. 2005). Furthermore it is known that habitats can be stratified by various abiotic and biotic factors. Therefore, I aimed to elucidate if effectively there is local adaptation due to salinity and investigated the effect of another biotic factor (*Vibrio spp*.) which is affected by salinity. The growth and virulence of *Vibrio spp*. increases with decreasing salinity (Larsen 1984, Wang 2005). Based on the result of the chosen response variables I partially found support for my main hypothesis of local adaptation due to salinity at spawning ground. In addition, I found strong phenotypic plasticity in Atlantic herring of the chosen populations.

The **first** hypothesis,

"Herring are locally adapted to the salinity at spawning grounds. Within population crosses show a better performance in their own salinity than the foreign within population cross",

found partially support in the response variables fertilization rate and mortality, as both variables showed an interaction of salinity and crossing. When comparing the two within population crosses, the Kiel cross had a maximal fertilization rate at 7 PSU, whereas the Danish cross showed a maximal fertilization rate at 7 and 20 PSU. Moreover, fertilization rate at 7 PSU was higher for the Kiel cross than for the Danish cross, but at 20 PSU no difference between the Danish and the Kiel cross were detected. While Kiel crosses had the highest fertilization in salinity resembling their own habitat, as predicted under a process of local adaptation, the Danish crosses had equal fertilization rates in both their own salinity and in the Kiel salinity. These findings may indicate a process of ongoing local adaptation, as for one population the "local vs. foreign" criterion is satisfied (Kawecki and Ebert, 2004). This diagnostic for local adaptation states that a local population "A" shows a higher fitness than a foreign population "B" in the habitat of population "A". In the Kiel cross we find support for this. The Danish cross, however, is performing equally well at 7 PSU as at 20 PSU and is not performing better at 20 PSU than the Kiel cross. The same pattern was also reflected in larval mortality. In 7 PSU the Kiel cross had a better survival than the Danish cross. However, in 20 PSU no survival difference was found for the two crosses (Kiel vs. Danish). A possible reason why Danish herring may not be as specifically adapted to the salinity at spawning ground like Kiel Canal herring could be due to the difference in hydrological features of both locations. The Kiel Canal is an artificially enclosed water body, where low fluctuation in salinity is expected. However, the spawning location in the Little Belt is much more exposed to fluctuation generated by the inflow of North Sea water and wind conditions. Salinity in the Kolding Fjord can vary annually from 14 to 23 PSU (Conley et al. 2000). It is suggested that in stable environments, where phenotypic plasticity is not essential (as it is also costly), selection for genetic adaptation on specific environmental factors is favored resulting in a decrease in phenotypic plasticity (Lande 2009). The Kiel Canal as a habitat may be more stable in respect to salinity than the Kolding Fjord and those impose a stronger selection on genetic adaptation to the development of the early life stages.

The **second** hypothesis,

"Between populations crosses show an intermediate performance compared to within population crosses, as the pattern of local adaptation is disrupted genetically",

found only confirmation in the fertilization rate. Both between population crosses show an equal performance at 7 and 20 PSU, even partly overlapping to 28 PSU. Especially, if KfKm and KfDm are compared, a "disruption" of the local adaptation pattern can be detected (Fig. 7). Hatching timing and mortality showed no such disruption pattern of local adaptation in the between population crosses. Hatching rate was driven by a maternal component, as in general crosses with a Kiel female (within and between population crosses) reached a higher hatching rate than crosses with a Danish female. Different factors could cause this effect. Firstly, Kiel Canal eggs may in general have a higher hatching rate than Danish eggs. Potentially, hatching rate could be confounded by the experimental design, as Kiel eggs were 24h younger than Danish eggs and may therefore be fresher and in a better quality. However, if this was the case, then this effect would have been expected in other response variables like fertilization rate. In general, the sparsely confirmation of the second hypothesis indicates that no strong pattern of local adaptation was identified in the selected populations. However, another response variable supporting local adaptation in Kiel Canal herring is the reduced fertilization rate of the cross DfKm compared to the other three crosses. This could indicate that sperm from Kiel Canal males are best adapted to fertilize eggs from Kiel females. Kroll et al (2013) revealed strong paternal effect on fertilization success of individual males in an Atlantic cod population. In Kiel Canal herring this paternal effect may not occur only on an individual but also on a population level. Meaning, that the Kiel Canal population may even be in a process of reproductive isolation. However, this effect could not be found into the other direction, in KfDm no reduced fertilization rate was found, implying that Kiel eggs do not perform better with population-specific sperms. Larvae from the DfKm cross hatched earlier than KfKm and KfDm larvae, however, this effect has to be interpreted with caution as DfKm had only three replicates and led to an unbalanced design and large error bars.

The **third** hypothesis,

"All crosses perform worst at a novel salinity not occurring in their natural spawning grounds",

was confirmed by all life-history traits. Fertilization rate decreased with higher salinity, and all crosses had the lowest fertilization rate at 28 PSU. High salinity delayed hatching timing, which is consistent with findings of the study from Griffin et al. (1998). They investigated hatching time in Pacific herring (*Clupea pallasi*) and found a delay in the highest salinity. Griffin et al. (1998) argue that the reason for this delay could lie in a resource allocation trade-off, as more energy is needed to maintain the ionic balance and therefore less energy is available for embryonic development. In my study, most losses of tanks (i.e. crossings) were as well in 28 PSU because full batches of larvae did not hatch. Larvae in this highest salinity had a shorter life-span (see Fig. 12). All this findings indicate an approach of the effective upper salinity tolerance limit for embryo development of Western Baltic herring. Nevertheless, as survival and performance of larvae in highest salinity was not zero, the tolerance to a wide salinity range in Atlantic herring is confirmed. *Clupea harengus* is

found to spawn in the northern hemisphere in salinities of 5-35 PSU (euryhaline species) (Holliday and Blaxter 1960). Colonization of fishes from the North Sea to the Baltic Sea is thought to have occurred mainly during the Littorina stage (7'500-4'000 years ago) (Segerstrale 1969, Ojaveer and Kalejs 2005). Therefore 28 PSU can be seen as the ancient salinity level of herring living nowadays in the Baltic Sea. However, different populations show individual ranges of salinity tolerance, especially in the eastern Baltic Sea, where speciation due to salinity created a herring subspecies (*Clupea harengus membras*) that is not capable to reproduce successfully at oceanic salinity anymore (Griffin et al. 1998).

The **fourth** hypothesis,

"Exposed to a bacterial stress, offspring from within population crosses of parents spawning in higher salinity show a reduced performance and immune response compared to offspring from parents spawning in low salinity",

could not be confirmed for every single aspects. Over all genes combined an effect of crossing on gene expression could be detected. The pattern was driven by a maternal component, showing different expression pattern for Kiel (KfKm and KfDm) versus Danish crosses (DfDm and DfKm). In the expression of the chosen immune genes again a crossing effect driven by a maternal component was detected. Especially the expression of the gene CC3 (complement component C3) was affected by crossing, being more upregulated in crosses with a Kiel female than in crosses with a Danish female. CC3 is the key protein for the activation of the complement cascade by e.g. enhancing phagocytosis of antigens and promoting inflammation. The upregulation of the CC3 gene indicates a higher activity of the complement system in crosses with a Kiel female. That would support the hypothesis that Kiel Canal herrings are exposed to a higher *Vibrio spp.* abundance and virulence because of low saline water and therefore enhance the activity of their complement system. Moreover, this result indicates that this specific immune information could be transferred via the mother, because immune gene expression pattern of the crosses differed solely in respect to female's origin. Løvoll et al (2007) found C3 protein in the unfertilised eggs of Atlantic salmon, suggesting a maternal transfer of C3-component via the egg. However, the downregulation of a single immune gene in Danish larvae cannot be converted to a "worse" performance of the whole immune system of Danish larvae. Moreover, if local adaptation to *Vibrio spp.* virulence on spawning sites would have occurred, an interaction of crossing and *Vibrio* on mortality would be expected, as different *Vibrio spp*. virulence and abundance are expected to occur at spawning sites, which differ in salinity level. The expression of the immune gene TNF2 (Tumor necrosis factor) was affected by an interaction of *Vibrio* and salinity. In the *Vibrio* treatment TNF2 was in general more down regulated in 20 PSU versus 7 PSU. TNF2 is a cell signalling protein involved in the inflammation response to inhibit the growth of pathogens. TNF2 may be less expressed in larvae of 20 PSU treatments, as *Vibrio* virulence is reduced in higher saline water and larvae need to invest less into immune response.

The **fifth** hypothesis,

"Between population crosses show an intermediate pattern or even a better immune response than within crosses, because offspring show a diversified immune competence, as their parents transfer information from different immune histories (different habitats= different pathogen exposure)"

was not confirmed. As already mentioned in hypothesis four, immune gene expression was driven by a maternal effect. So, between populations crosses had not an influence on the ability of larvae to cope with a *Vibrio spp*. stress. For the expression pattern of the epigenetic genes again a crossing effect could be identified. Especially for genes involved in silencing a clustering of KfDm apart from the three other crosses was detected. Moreover, HDAC1 (involved in deacetylation) was more downregulated in KfDm versus both Danish crosses (DfDm and DfKm). This may indicate the transfer of epigenetic information from Kiel mothers to offspring, however, the expression of HDAC1 in the KfKm cross versus all other crosses did not differ. The expression pattern of epigenetic genes involved in gene activation yielded an interaction of salinity and *Vibrio*, such that the 7 PSU control treatment clustered apart from both *Vibrio* treatment (7 and 20 PSU) and the 20 PSU control treatment. Moreover, the gene TPR (involved in demethylation) showed an interaction of salinity and *Vibrio*; in the *Vibrio* treatment TPR was upregulated at 7 PSU but downregulated at 20 PSU. This epigenetic factor seems to be important in handling salinity and *Vibrio* stress. Epigenetic genes are known to be affected by environmental factors. It was observed that these informations can be passed on to the next generation affecting the phenotypic plasticity of the offspring and if such patterns become genetically assimilated, they can contribute to local adaptation of populations (Jablonka and Lamb 1998, 2015). Overall the stress genes showed no pattern, but the expression of the single stress gene hspDNAj4 was affected by crossing. In the between population cross KfDm hspDNAj4 was more upregulated than in DfKm. This gene expression pattern shows a trend to a maternal compound, with an upregulation in Danish crosses and a downregulation in Kiel crosses.

The **sixth** hypothesis,

"Offspring of populations of low saline water are better adapted to a Vibrio spp. stress and should therefore be better in coping with a combination of the two stressors (salinity and pathogen) than population of higher saline waters",

was not confirmed. The three-fold interaction of the factors on mortality was significant, but did not reveal a significant better survival for Kiel crosses versus Danish crosses exposed to *Vibrio* across the different salinity levels. Therefore parent's origin did not matter in the ability to cope with a combination of both stressors. A significant interaction between *Vibrio* and salinity on mortality was detected. Virulence of *Vibrio spp.* increased in lower salinity (Larsen 1984, Wang 2005) as indicated by a higher mortality of larvae in 7 and 20 PSU than in 28 PSU. At 28 PSU the virulence of *Vibrio spp.* seems to vanish, as survival was equal between control and *Vibrio* treatment. As no interaction between *Vibrio* and crossing was found, it can be argued that none of the crosses was better in coping with a *Vibrio spp*. stress. Eiler et al. (2006) showed that the distribution pattern of different *Vibrio* species along a salinity gradient in the Baltic Sea (Swedish coastline) depends on several

factors. For example*, Vibrio alginolyticus* had a higher abundance in high than low saline waters, but *Vibrio anguillarum* showed the opposite pattern. As herrings show a migratory behaviour, it can be assumed that individuals experience high *Vibrio* diversity, and have to cope with isolates of different abundances and virulences. This may explain the absence of an interaction between *Vibrio* treatment and crossing, as adult herring have the chance to build up a diversified immune competence and immune memory and transfer this immunological information to their offsprings. The *Vibrio* isolate used in this study was a strain from Italy and supposed to be allopatric for both populations.

Finally, the virulence increase of *Vibrio spp.* in low salinity was confirmed by the mortality data. Larvae showed a shorter life-span in 7 and 20 PSU than in the highest PSU, where virulence seems to vanish. This result shows how two factors (biotic and abiotic) coming into an interaction, can affect the survival of early life stages. However, the higher virulence in low salinity of *Vibrio spp.* is not reflected in gene expression pattern, e.g. in the immune gene expression. Either, I overlooked the effect of *Vibrio spp.* on the immune system by not selecting the right genes or because the chosen bacteria strain was new to the larvae and one day sampling after the treatment was too early to detect an effect. Moreover, mortality started on a later stage as gene expression samples were taken. For a more comprehensive view on the immune system, it would be necessary to conduct cellular immune assays in addition. However, this could not be done in the framework of this thesis, because of experimental constrains.

Importantly, it has to be mentioned that the survival data (beaker dataset) are a combination of pseudo-replicate (5 larvae in a beaker) and true biological replicate (up to 8 replicate per treatment). Due to the small number of larvae in a beaker it was not possible to create a “mean survival curve” or nesting families within treatment. Every individual was thus treated as an independent data point. The number of available larvae was not sufficient to follow a different approach, and keeping larvae singly was not possible due to high number of larvae used. I am aware that in future experiments, more larvae per tank should be used such that mean survival per tank can be calculated.

6 Conclusion

In my master thesis, I detected strong phenotypic plasticity in herring, with signs for ongoing local adaptation to the spawning ground in the Kiel Canal. In the context of climate change, I suggest that not the decline of salinity will be the main stressor for Western Baltic herring, but rather another stressor that is affected by salinity: the virulence of *Vibrio* pathogens. The increase of pathogen virulence (here *Vibrio spp*.) by decreasing salinity will probably be one of the main stressors, moreover, warmer temperature (climate warming) as well affects *Vibrio spp*. growth (Oberbeckmann et al. 2012, Larsen 2004). The suggestion of local adaptation to salinity on spawning sites in herring was until now based on neutral genetic markers (Bekkevold et al. 2005, Gaggiotti et al. 2009). The results of this study give a new perspective based on phenotypic traits on the potential of local adaptation processes in Western Baltic herring.

Outlook- I see this investigations as a pilot study to elucidate local adaptation in herring. To determine properly a spatial scale of local adaptation in Western Baltic spring-spawning herring, it would be important in further experiments to include several populations along a salinity gradient (e.g. Greifswalder Bodden, inner Danish waters, Swedish west coast/Skagerrak). Moreover, it would be necessary to confirm the origin of adult herring via microsatellites (Ruzzante et al 2006). To gain a better insight if epigenetic genes are a way for males to transfer paternal effects, a transgenerational approach would be more appropriate. Although, the rearing of adult herring is known to be quite difficult, adult individuals would be acclimatized in different salinity levels and offspring from each group then exposed to all salinity levels. Such an approach would help to disentangle epigenetic, genetic and environmental effects.

7 Acknowledgements

Merci beaucoup to…

… Olivia Roth, for giving me the possibility to work on that nice project and your confidence that enables me to work with a large independency and let me “learning by doing”!

… Anne Beemelmanns and Andrea Franke, for experimentally, laboratory and statistical advices.

… Kathrin, for the nice lunch times and your friendship.

… my family and friends in Switzerland for your support (and chocolate).

… Mathis, for simply being here, anytime.

8 Appendix

Table 8.1 Primer sequences of epigenetic genes and primer assay efficiencies in % and R^2

Gene name	Primer forward 5'-3'	Primer reversed 5'-3'	Amplicon length	Efficiency %	R^2
Bromo	TGGAACCAGTAAAGAAGTCCGA	TGGTCACATAGTAGCGGTTCTT	108	111.7	0.903
Pcaf	CCAGACCATGTTTGAGCTGAGT	ATGTGGAGCCGTCATCTTTCTG	110	93.8	0.881
Moz	TGGACAACGTTCCCCTGAAG	CAGAGAAGCCGCTGTCAGAT	99	123.5	0.993
HDAC1	GATGGCATTGACGACGAATCTT	ACGGCAGGTTAAAGCTCTTCAT	196	98	0.960
SPRY	GATTACGTGGACAAGGCAGAGA	TGGCTCACACCGTTCTTGTAG	80	96.7	0.890
TPR	GTAAACCATACCGAGGCCGT	AAGGCTGTTCGCTGCTATGA	198	108	0.970
RFD	ATGAGGCCCGAGTCAAATGG	GAGCTGCCTCAGCACAAAAT	80	107.5	0.950
ADDZ	TCAGTATGACGACGATGGCTAC	ACCCACTAACAGATCCACACAC	127	93.8	0.960

Table 8.2 Post hoc analysis (Tukey's test) of the interaction salinity*crossing on fertilization rate from the within population crosses (KfKm and DfDm). Abbreviations: K= Kiel, D= Danish, f= female, m= male ; * denotes a significant result (p<0.05), . denotes a trend (p<0.1)

Post hoc test	P
Crossing*Salinity	
DfDm, 7 PSU- DfDm, 20 PSU	0.9982
DfDm, 7 PSU- DfDm, 28 PSU	**0.0162***
DfDm, 20 PSU- DfDm, 28 PSU	**0.0345***
KfKm, 7 PSU- KfKm, 20 PSU	0.0919 .
KfKm, 7 PSU- KfKm, 28 PSU	**0.0000*****
KfKm, 20 PSU- KfKm, 28 PSU	**0.0000*****
DfDm, 7 PSU- KfKm, 7 PSU	**0.0103***
DfDm, 7 PSU- KfKm, 20 PSU	0.9241
DfDm, 7PSU- KfKm, 28 PSU	**0.0003*****
DfDm, 20 PSU- KfKm, 7 PSU	**0.0021****
DfDm, 20 PSU- KfKm, 20 PSU	0.7047
DfDm, 20 PSU- KfKm, 28 PSU	**0.0007*****
DfDm, 28 PSU- KfKm, 7 PSU	**0.0000*****
DfDm, 28 PSU- KfKm, 20 PSU	**0.0008*****
DfDm, 28 PSU- KfKm, 28 PSU	0.9006

Table 8.3 Post hoc analysis (Tukey's test) of salinity and the interaction crossing*salinity on survival analysis (tank dataset). Abbreviations: K= Kiel, D= Danish, f= female, m= male ; * denotes a significant result (p<0.05), . denotes a trend (p<0.1)

Post hoc test	P
Salinity	
7PSU- 20 PSU	**<0.001*****
7 PSU- 28 PSU	**<0.001*****
20 PSU- 28 PSU	**<0.001*****
Crossing*Salinity	
DfDm, 7 PSU – DfDm, 20 PSU	1.000
DfDm, 7 PSU – DfDm, 28 PSU	**<0.001*****
DfDm, 20 PSU – DfDm, 28 PSU	**<0.001*****
DfDm, 7 PSU – DfKm, 7 PSU	0.9959
DfDm, 7 PSU – DfKm, 20 PSU	0.8620
DfDm, 7 PSU – DfKm, 28 PSU	0.9999

DfDm, 7 PSU – KfDm, 7 PSU	0.0036
DfDm, 7 PSU – KfDm, 20 PSU	0.7911
DfDm, 7 PSU – KfDm, 28 PSU	**<0.001*****
DfDm, 7 PSU – KfKm, 7 PSU	0.0523
DfDm, 7 PSU – KfKm, 20 PSU	0.1692
DfDm, 7 PSU – KfKm, 28 PSU	**<0.001*****
DfDm, 20 PSU – DfKm, 7 PSU	0.9993
DfDm, 20 PSU – DfKm, 20 PSU	0.5624
DfDm, 20 PSU – DfKm, 28 PSU	0.9999
DfDm, 20 PSU – KfDm, 7 PSU	0.0015381
DfDm, 20 PSU – KfDm, 20 PSU	0.3687
DfDm, 20 PSU – KfDm, 28 PSU	**<0.001*****
DfDm, 20 PSU – KfKm, 7 PSU	0.0360
DfDm, 20 PSU – KfKm, 20 PSU	0.0173
DfDm, 20 PSU – KfKm, 28 PSU	**<0.001*****
DfDm, 28 PSU – DfKm, 7 PSU	**<0.001*****
DfDm, 28 PSU – DfKm, 20 PSU	**<0.001*****
DfDm, 28 PSU – DfKm, 28 PSU	**<0.001*****
DfDm, 28 PSU – KfDm, 7 PSU	**<0.001*****
DfDm, 28 PSU – KfDm, 20 PSU	**<0.001*****
DfDm, 28 PSU – KfDm, 28 PSU	**0.0109****
DfDm, 28 PSU – KfKm, 7 PSU	**<0.001*****
DfDm, 28 PSU – KfKm, 20 PSU	**<0.001*****
DfDm, 28 PSU – KfKm, 28 PSU	0.9969
DfKm, 7 PSU – DfKm, 20 PSU	**0.0243***
DfKm, 7 PSU – DfKm, 28 PSU	0.9958
DfKm, 20 PSU – DfKm, 28 PSU	1.000
DfKm, 7 PSU – KfDm, 7 PSU	**0.0016*****
DfKm, 7 PSU – KfDm, 20 PSU	**<0.001*****
DfKm, 7 PSU – KfDm, 28 PSU	**<0.001*****
DfKm, 7 PSU – KfKm, 7 PSU	0.0678 .
DfKm, 7 PSU – KfKm, 20 PSU	**<0.001*****
DfKm, 7 PSU – KfKm, 28 PSU	**<0.001*****
DfKm, 20 PSU – KfDm, 7 PSU	**<0.001*****
DfKm, 20 PSU – KfDm, 20 PSU	1.000
DfKm, 20 PSU – KfDm, 28 PSU	**<0.001*****
DfKm, 20 PSU – KfKm, 7 PSU	**<0.001*****
DfKm, 20 PSU – KfKm, 20 PSU	0.9975
DfKm, 20 PSU – KfKm, 28 PSU	**<0.001*****
DfKm, 28 PSU – KfDm, 7 PSU	0.4964
DfKm, 28 PSU – KfDm, 20 PSU	1.000
DfKm, 28 PSU – KfDm, 28 PSU	0.3809
DfKm, 28 PSU – KfKm, 7 PSU	0.7339
DfKm, 28 PSU – KfKm, 20 PSU	0.9999
DfKm, 28 PSU – KfKm, 28 PSU	**0.0001*****
KfDm, 7 PSU – KfDm, 20 PSU	**<0.001*****
KfDm, 7 PSU – KfDm, 28 PSU	**<0.001*****
KfDm, 20 PSU – KfDm, 28 PSU	**<0.001*****
KfDm, 7 PSU – KfKm, 7 PSU	0.9831
KfDm, 7 PSU – KfKm, 20 PSU	**<0.001*****
KfDm, 7 PSU – KfKm, 28 PSU	**<0.001*****
KfDm, 20 PSU – KfKm, 7 PSU	**<0.001*****

KfDm, 20 PSU – KfKm, 20 PSU	0.9289
KfDm, 20 PSU – KfKm, 28 PSU	**<0.001*** **
KfDm, 28 PSU – KfKm, 7 PSU	**<0.001*** **
KfDm, 28 PSU – KfKm, 20 PSU	**<0.001*** **
KfDm, 28 PSU – KfKm, 28 PSU	**0.0043** **
KfKm, 7 PSU – KfKm, 20 PSU	**<0.001*** **
KfKm, 7 PSU – KfKm, 28 PSU	**<0.001*** **
KfKm, 20 PSU – KfKm, 28 PSU	**<0.001*** **

Table 8.4 Post hoc analysis (Tukey's test) of salinity and the interactions crossing*salinity, Vibrio*salinity on survival analysis (beaker dataset). Abbreviations: K= Kiel, D= Danish, f= female, m= male, C=Control, V=Vibrio ; * denotes a significant result (p<0.05), . denotes a trend (p<0.1)

Post hoc test	P
Salinity	
7PSU- 20 PSU	0.9995 ns
7 PSU- 28 PSU	0.0593 .
20 PSU- 28 PSU	0.0519 .
Crossing*Salinity	
DfDm, 7 PSU – DfDm, 20 PSU	1.000
DfDm, 7 PSU – DfDm, 28 PSU	0.5136
DfDm, 20 PSU – DfDm, 28 PSU	0.5975
DfDm, 7 PSU – DfKm, 7 PSU	0.9981
DfDm, 7 PSU – DfKm, 20 PSU	1.000
DfDm, 7 PSU – DfKm, 28 PSU	0.2259
DfDm, 7 PSU – KfDm, 7 PSU	1.000
DfDm, 7 PSU – KfDm, 20 PSU	0.1581
DfDm, 7 PSU – KfDm, 28 PSU	0.6359
DfDm, 7 PSU – KfKm, 7 PSU	0.0766 .
DfDm, 7 PSU – KfKm, 20 PSU	0.9887
DfDm, 7 PSU – KfKm, 28 PSU	0.9619
DfDm, 20 PSU – DfKm, 7 PSU	0.9998
DfDm, 20 PSU – DfKm, 20 PSU	1.000
DfDm, 20 PSU – DfKm, 28 PSU	0.2782
DfDm, 20 PSU – KfDm, 7 PSU	1.000
DfDm, 20 PSU – KfDm, 20 PSU	0.1891
DfDm, 20 PSU – KfDm, 28 PSU	0.7259
DfDm, 20 PSU – KfKm, 7 PSU	0.0896
DfDm, 20 PSU – KfKm, 20 PSU	0.9975
DfDm, 20 PSU – KfKm, 28 PSU	0.9846
DfDm, 28 PSU – DfKm, 7 PSU	0.8829
DfDm, 28 PSU – DfKm, 20 PSU	0.4563
DfDm, 28 PSU – DfKm, 28 PSU	0.9999
DfDm, 28 PSU – KfDm, 7 PSU	0.4429
DfDm, 28 PSU – KfDm, 20 PSU	1.000
DfDm, 28 PSU – KfDm, 28 PSU	0.9999
DfDm, 28 PSU – KfKm, 7 PSU	1.000
DfDm, 28 PSU – KfKm, 20 PSU	0.9308
DfDm, 28 PSU – KfKm, 28 PSU	0.9984
DfKm, 7 PSU – DfKm, 20 PSU	0.9973
DfKm, 7 PSU – DfKm, 28 PSU	0.5811

DfKm, 20 PSU – DfKm, 28 PSU	0.1771
DfKm, 7 PSU – KfDm, 7 PSU	0.9965
DfKm, 7 PSU – KfDm, 20 PSU	0.5448
DfKm, 7 PSU – KfDm, 28 PSU	0.9738
DfKm, 7 PSU – KfKm, 7 PSU	0.3233
DfKm, 7 PSU – KfKm, 20 PSU	1.000
DfKm, 7 PSU – KfKm, 28 PSU	0.9999
DfKm, 20 PSU – KfDm, 7 PSU	1.000
DfKm, 20 PSU – KfDm, 20 PSU	0.0757 .
DfKm, 20 PSU – KfDm, 28 PSU	0.5284
DfKm, 20 PSU – KfKm, 7 PSU	**0.0295***
DfKm, 20 PSU – KfKm, 20 PSU	0.9816
DfKm, 20 PSU – KfKm, 28 PSU	0.9518
DfKm, 28 PSU – KfDm, 7 PSU	0.1693
DfKm, 28 PSU – KfDm, 20 PSU	0.9999
DfKm, 28 PSU – KfDm, 28 PSU	0.9931
DfKm, 28 PSU – KfKm, 7 PSU	0.9999
DfKm, 28 PSU – KfKm, 20 PSU	0.6711
DfKm, 28 PSU – KfKm, 28 PSU	0.9653
KfDm, 7 PSU – KfDm, 20 PSU	0.0699 .
KfDm, 7 PSU – KfDm, 28 PSU	0.5099
KfDm, 20 PSU – KfDm, 28 PSU	0.9999
KfDm, 7 PSU – KfKm, 7 PSU	**0.0268***
KfDm, 7 PSU – KfKm, 20 PSU	0.9781
KfDm, 7 PSU – KfKm, 28 PSU	0.9265
KfDm, 20 PSU – KfKm, 7 PSU	1.000
KfDm, 20 PSU – KfKm, 20 PSU	0.6647
KfDm, 20 PSU – KfKm, 28 PSU	0.9966
KfDm, 28 PSU – KfKm, 7 PSU	0.9982
KfDm, 28 PSU – KfKm, 20 PSU	0.9917
KfDm, 28 PSU – KfKm, 28 PSU	0.9999
KfKm, 7 PSU – KfKm, 20 PSU	0.4253
KfKm, 7 PSU – KfKm, 28 PSU	0.9802
KfKm, 20 PSU – KfKm, 28 PSU	0.9999
Vibrio*Salinity	
7 PSU, C - 20 PSU, C	0.2302
7 PSU, C - 28 PSU, C	0.6086
20 PSU, C - 28 PSU, C	0.9999
7 PSU, V - 7 PSU, C	**0.0000*****
20 PSU, V - 7 PSU, C	**0.0000*****
28 PSU, V - 7 PSU, C	0.5365
7 PSU, V - 20 PSU, C	**0.0000*****
20 PSU, V - 20 PSU, C	**0.0021****
28 PSU, V - 20 PSU, C	0.9999
7 PSU, V - 28 PSU, C	**0.0000*****
20 PSU, V - 28 PSU, C	**0.0170***
28 PSU, V - 28 PSU, C	0.9999
7 PSU, V – 20 PSU, V	0.2500
7 PSU, V – 28 PSU, V	**0.0000*****
20 PSU, V – 28 PSU, V	**0.0241***

Table 8.5 Number of replicates of each treatment group used for gene expression analysis. Abbreviations: K= Kiel, D= Danish, f= female, m= male

Control			Vibrio	
	7 PSU	20 PSU	7 PSU	20 PSU
DfDm	7	3	6	4
DfKm	5	4	5	5
KfDm	4	7	4	4
KfKm	5	7	6	6

Table 8.6 PERMANOVA on gene expression of gene groups. * denotes a significant result (p<0.05), . denotes a trend (p<0.1)

PERMANOVA			
Immune genes	df	R^2	P
Crossing	3	0.0817	**0.018 ***
Salinity	1	0.0142	0.278
Vibrio	1	0.0045	0.823
Basic cell function	df	R^2	P
Crossing	3	0.0740	0.086 .
Salinity	1	0.0009	0.922
Vibrio	1	0.0027	0.776
Epigenetic genes (activation)	df	R^2	P
Crossing	3	0.0483	0.232
Salinity	1	0.0024	0.891
Vibrio	1	0.0073	0.557
Crossing*Salinity	3	0.0136	0.928
Crossing*Vibrio	3	0.0175	0.835
Salinity*Vibrio	1	0.0501	**0.013 ***
Crossing*Salinity*Vibrio	3	0.0643	0.102
Epigenetic genes (silencing)	df	R^2	P
Crossing	3	0.1112	**0.022 ***
Salinity	1	0.0053	0.523
Vibrio	1	0.0043	0.563
Osmoregulation genes	df	R^2	P
Crossing	3	0.0873	0.056 .
Salinity	1	0.0171	0.211
Vibrio	1	0.0154	0.249
Stress genes	df	R^2	P
Crossing	3	0.0599	0.083 .
Salinity	1	0.0091	0.524
Vibrio	1	0.0017	0.968

Table 8.7 ANOVA on gene expression of single genes with a significant result. * denotes a significant result (p<0.05), . denotes a trend (p<0.1)

ANOVA			
CC3 (immune gene)	df	F-value	P
Crossing	3	5. 967	**0.0014 ****
Salinity	1	1.666	0.2006
Vibrio	1	0.020	0.8881
HDAC1 (epigenetic gene)	df	F-value	P
Crossing	3	3.299	**0.0248 ***
Salinity	1	0.299	0.5859
Vibrio	1	0.390	0.5341
hspDNAj4 (stress gene)	df	F-value	P
Crossing	3	3.669	**0.0158 ***
Salinity	1	0.023	0.8790
Vibrio	1	0.029	0.8660
TNF2 (immune gene)	df	F-value	P
Crossing	3	2.021	0.1182
Salinity	1	3.396	0.0693 .
Vibrio	1	0.098	0.7556
Vibrio*Salinity	1	4.666	**0.0340 ***
TPR (epigenetic gene)	df	F-value	P
Crossing	3	1.384	0.2555
Salinity	1	0.115	0.7360
Vibrio	1	0.123	0.72677
Crossing*Salinity	3	0.404	0.75062
Crossing*Vibrio	3	0.817	0.48887
Vibrio*Salinity	1	7.892	**0.00653 ****
Crossing*Salinity*Vibrio	3	2.771	**0.04837 ***

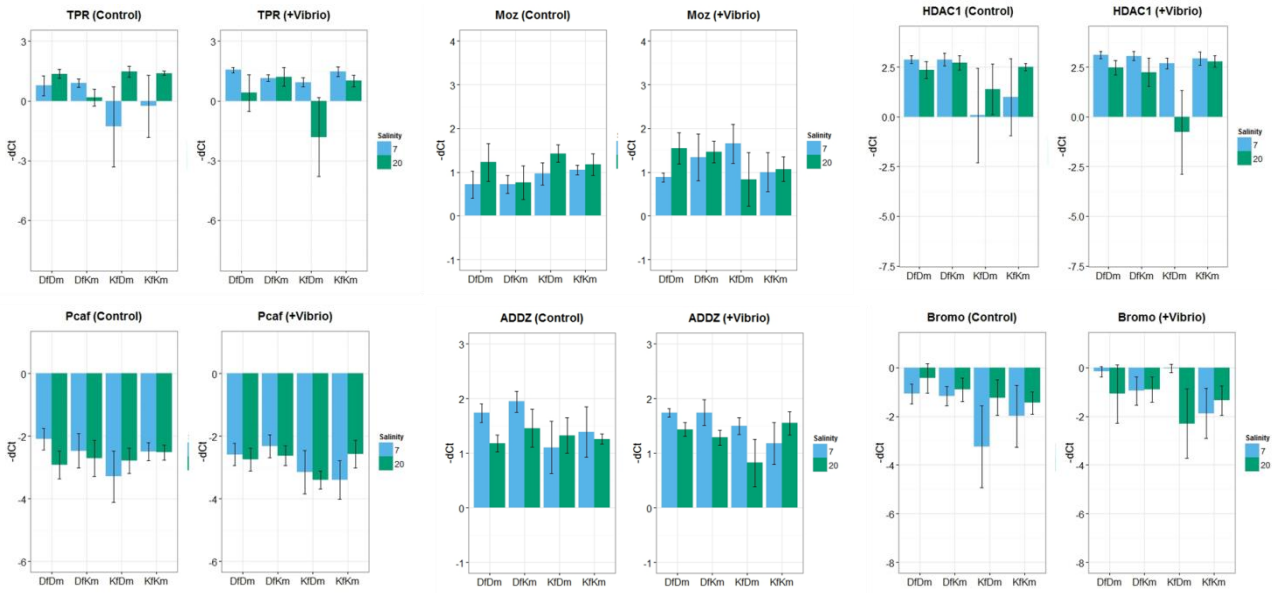
Table 8.8 Post hoc analysis (Tukey's test) on gene expression of single genes. * denotes a significant result (p<0.05), . denotes a trend (p<0.1)

Tukey post hoc	
CC3 (immune gene)	P
DfKm- DfDm	0.8515
KfDm-DfDm	**0.0014 ****
KfKm-DfDm	**0.0419 ***
KfDm-DfKm	**0.0195 ***
KfKm-DfKm	0.2732
KfKm-KfDm	0.5431
HDAC1 (epigenetic gene)	
DfKm- DfDm	0.9997
KfDm-DfDm	**0.0329 ***
KfKm-DfDm	0.9088
KfDm-DfKm	**0.0451 ***
KfKm-DfKm	0.9418
KfKm-KfDm	0.1186
hspDNAj4 (stress gene)	
DfKm- DfDm	0.6727

KfDm-DfDm	0.1712
KfKm-DfDm	0.6742
KfDm-DfKm	**0.0118 ***
KfKm-DfKm	0.1094
KfKm-KfDm	0.7199
TNF2 (immune gene)	
7 PSU, C - 20 PSU, C	0.9987
7 PSU, V - 7 PSU, C	0.3423
7 PSU, C - 20 PSU, V	0.6171
7 PSU, V – 20 PSU, C	0.2663
20 PSU, V - 20 PSU, C	0.7099
7 PSU, V - 20 PSU, V	**0.0278 ***
TPR (epigenetic gene)	
7 PSU, C - 20 PSU, C	0.2243
7 PSU, V - 7 PSU, C	0.1371
7 PSU, C - 20 PSU, V	0.9882
7 PSU, V – 20 PSU, C	0.9942
20 PSU, V - 20 PSU, C	0.4093
7 PSU, V - 20 PSU, V	0.2795

Fig 8.1 Interaction of salinity and Vibrio on relative gene expression (–δCt). Basic cell function genes (top six plots) and and immune genes

Epigenetic genes



Stress genes

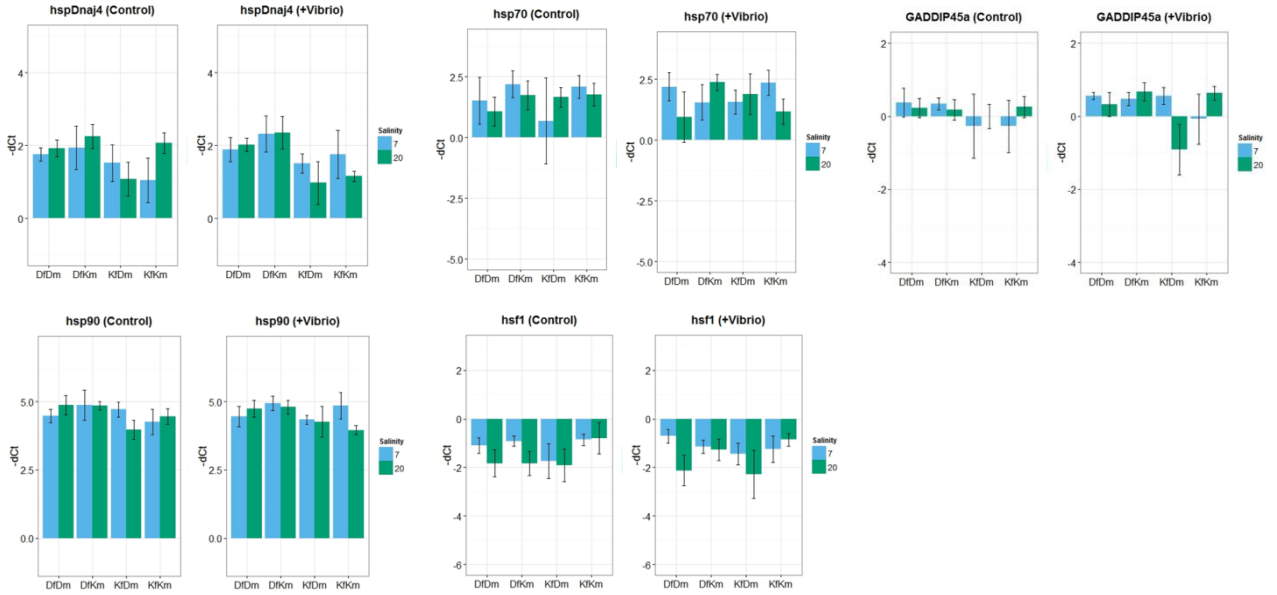
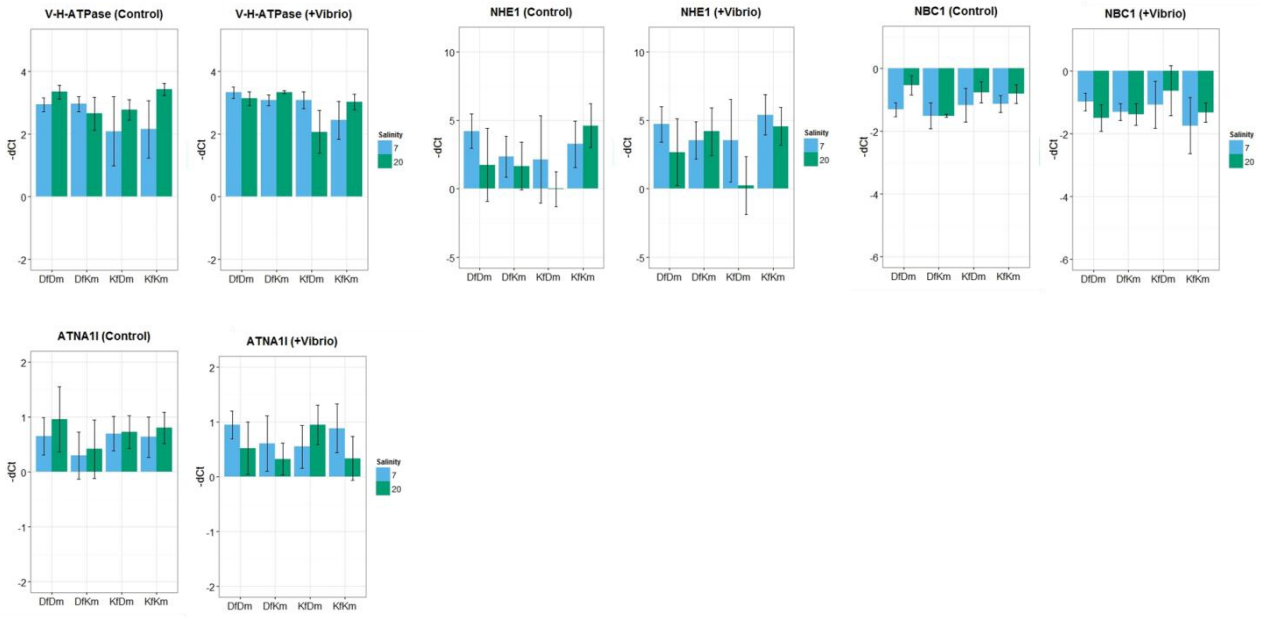


Fig 8.2 Interaction of salinity and Vibrio on relative gene expression (–δCt). Epigenetic genes and and stress genes

Osmoregulation genes



Metabolism gene

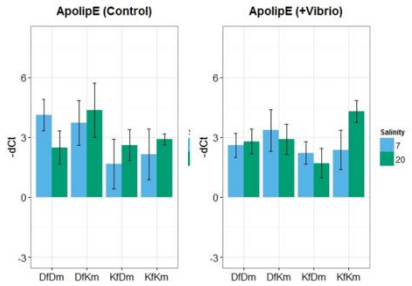


Fig 8.3 Interaction of salinity and Vibrio on relative gene expression (−δCt). Osmoregulation genes and metabolism gene

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10 Declaration of authorship

Herewith I certify that the present thesis, apart from the consultation of my supervisor, was independently prepared by me. No other than the indicated resources and references were used. This thesis was presented to no other place within the scope of an examination procedure. The written thesis is identical with the electronic one.

Kiel, 30th November 2015

Maude Poirier